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(54) **Partially double-stranded oligonucleotide and method for forming oligonucleotide**

Partiell doppelsträngiges Oligonukleotid und Verfahren zu seiner Bildung

Oligonucléotide à double brin partiel et son procédé de formation

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Description**BACKGROUND OF THE INVENTION**5 **Field of the Invention**

This invention relates to a novel method for separating a target oligonucleotide.

Also, the present invention relates to a formation method which can be suitably used for detecting a target oligonucleotide.

10 Further, the present invention relates to a method for imparting a capturing function to an oligonucleotide.

Related Background Art

Owing to the development in genetic manipulation technology in recent years, it has become possible to obtain a protein which is the genetic product in a large amount by cloning a specific gene and having that expressed in *E. coli*, *B. subtilis*, etc. According to this method, since the protein is produced in a microorganism of which growth is rapid in growth, there is an advantage that even a protein which can be in itself purified and separated with difficulty by enzymes can be obtained easily and yet in a large amount, whereby the cost can be reduced in industry.

However, on the other hand, it does not necessarily follow that any protein can be well expressed by the same method. In such a case, there are many examples where expression has been rendered possible by replacing the base sequence of the structural gene of a desired protein with, for example, the codon well used in *E. coli* to constitute a synthetic gene.

Also, there have been recently made attempts to modify proteins for examination of the relationship of their functions with the amino acid sequences, or for making their functions higher. In such a case, it has been frequently practiced that a gene is constructed by introducing previously a restriction enzyme cleavage site as far as possible into the structural gene of the protein without changing the amino acid sequence, synthesizing and expressing it, followed by replacement of a part by utilizing the restriction enzyme cleavage site introduced to modify the protein.

Presently, among the synthetic methods of gene (hereinafter referred to as double-stranded oligonucleotide) practiced, for example, there is a method in which a respective chains of the desired DNA are separated into 100 mers or less, which are synthesized by a DNA synthesizer and formed into a double strand by annealing, or after they are formed into double strands, ligated so as to be arranged consecutively. To describe specifically, when a double-stranded oligonucleotide of 100 base pairs is desired to be synthesized, two oligonucleotides of 100 mers are synthesized, then the oligonucleotides complementary to each other are mutually annealed to form a double strand, or when a double-strand of 200 base pairs is desired to be synthesized, 4 oligonucleotides of 100 mers are synthesized, and then the oligonucleotides complementary to each other are mutually annealed to form a double strand, followed by ligation of two sets of said double strands. When a gene of 500 base pairs is desired to be obtained, 10 oligonucleotides of 100 mers are synthesized, and then the oligonucleotides complementary to each other are mutually annealed to form double strands, followed by ligation successively, etc.

In preparing such a double-stranded oligonucleotide if its length is 100 mer or less, in place of forming two single-stranded oligonucleotides complementary to each other and forming a double strand by annealing, a primer can be synthesized and a double strand can be formed according to the primer extension method.

More specifically, in the case of synthesizing a double-stranded oligonucleotide of 100 base pairs, one oligonucleotide of 100 mers is synthesized, and by using it as a template, and also bonding it to an oligonucleotide of 8 bases or more having a sequence complementary to said oligonucleotide as the primer, nucleic acid bases can be polymerized successively from the primer side.

On the other hand, as the representative DNA labelling methods, there have been known those utilizing (1) the end labelling method, (2) the nick translation method, (3) the substitution synthetic method, and (4) the primer extension method.

In the following, the respective methods for obtaining labelled double-stranded oligonucleotide are to be described.

50 The end labelling method is a method in which the phosphoric acid group at the 5'-end of oligonucleotide is eliminated with alkali phosphatase, and a polynucleotide kinase is allowed to act thereon to label it during phosphorylating it again. The end labelling method is utilized for radioactive labelling with ³²P. On the other hand, for labelling of the 3'-end, there are the terminal transferase method, the DNA polymerase method, etc. The terminal transferase method is a method in which a number of nucleotide triphosphates labelled with an enzyme are bonded successively at the 3'-end, while the DNA polymerase method is a method in which the protrudent single strand portion of the DNA cleaved with a restriction enzyme is modified, and the labelled nucleotide is incorporated during formation of a complementary double strand. Since in the end labelling method the site to be thus modified is limited to about one or two places, it is difficult to obtain a probe with high specific activity. Generally, labelling with radioisotope having high specific activity

is performed.

In contrast, the nick translation method and the substitution synthetic method can prepare a DNA probe having high specific activity by incorporation of a large number of labelling compounds. In short, in the nick translation method, a double strand of DNA is hydrolyzed randomly with DNase I derived from pancreas to form nicks. DNA polymerase I recognizes the nicks and decomposes the DNA chain from the 5'-side, and at the same time a complementary DNA is synthesized with the DNA chain having no nick as the template by the polymerase activity in the direction from the 5'-side to the 3'-side, whereby the labelled nucleotide is incorporated as the substrate. On the other hand, the substitution synthetic method is a method in which the both 3'-ends of the double strand are cut in suitable lengths by 3'-exonuclease activity of T4 DNA polymerase, and then the labelled nucleotide is incorporated during modification by polymerase activity. Both of said methods are effective only when labelling long DNA of some hundred base pairs or more.

The primer extension method is a method in which there is utilized the Sanger method for determining the base sequence of DNA, and in which a single-stranded DNA oligonucleotide which becomes the template is synthesized, its 3'-end is bonded to an oligonucleotide of 8 bases or more having a complementary sequence as the primer, and the labelled nucleotide is incorporated during synthesis of DNA having a complementary sequence from the 5'-side to the 3'-side with a large fragment of DNA polymerase I, etc.

The synthesizing ability of a commercially available DNA synthesizer is presently about 100 mers or less. Besides, the yield cannot be said to be high. Accordingly, the oligonucleotide synthesized is separated by gel electrophoresis, etc. and the only desired band must be extracted. For example, in the case as described above (when synthesizing a double-stranded oligonucleotide of 200 base pairs), 4 oligonucleotides of 100 mers are synthesized, and further purified before use. Also, in the case of synthesizing a double-stranded oligonucleotide of 500 base pairs, 10 oligonucleotides of 100 mers are synthesized, and further purified before use.

Next, these oligonucleotides are annealed to form double strands, which are in turn ligated. In this case, and there are two kinds of oligonucleotides in respect of the binding order, the two sets of AB and BA. Therefore, for the bound products, by determining the base sequence, only the order of AB is required to be selected. Necessarily, two kinds of bound products are obtained in the both directions, and the operation for obtaining only the desired product by determining the base sequences thereof is required. However, the above operation is considerably complicated, and yet when purification is performed for satisfying the purity, the synthesis yield becomes poor. Thus, this method was not satisfactory in both purity and yield.

Also, as described above, even if a double-stranded oligonucleotide may be formed by the primer extension method, etc., the maximum number of base pairs of the double-stranded oligonucleotide before ligation by annealing is up to 100 (because the synthesis ability of single-stranded oligonucleotide is presently 100 mers or less), and there has not been obtained a method of synthesizing a double-stranded oligonucleotide of 200 base pairs at once without the operation of ligation.

On the other hand, generally speaking, as the length of the DNA oligonucleotide utilized as the probe is longer, the error of complementary bonding during hybridization can be prevented. However, for obtaining a long labelled DNA according to the primer extension method, a long single-stranded DNA is required which becomes the template. Whereas, the synthesis yield by a DNA synthesizer is lowered, as the length of the oligonucleotide to be synthesized is longer. Besides, also the by-products with shorter chain lengths are increased, and therefore the desired product is required to be purified by separation in order to utilize it as the template DNA.

Also, in the case of synthesizing a long region with DNA polymerase, synthesis may be sometimes stopped in the course by some obstacle, thereby giving rise to difference in length of the labelled oligonucleotides. Particularly, when a nonradioactive labelling substance is to be incorporated, the side chain of the labelling substance, etc. interferes with the enzyme reaction, whereby products with shorter lengths than those expected previously are liable to be formed. This not merely lowers the efficiency of hybridization, but also affects the reaction conditions of hybridization (temperature, formamide content, etc.), and may probably result in unsuccessful hybridization reaction itself.

Further, according to this method, one chain of the double strand functions only as the template, and the chain which is labelled and functions as the probe in the hybridization reaction mixture is only one on the primer side. Therefore, it follows that only half of all DNAs is utilized, whereby efficiency is poor. Further, during the hybridization reaction, the unlabelled template DNA may antagonize the labelled probe, whereby there is the possibility that the detection sensitivity may be lowered.

This problem applies to all the probes synthesized by the primer extension method irrespectively of the length of the probe, and a large amount of probes is required in a system where high sensitivity is demanded, and further in some cases, the operation of recovering only the template portion having no label may become required.

Also, during ligation by annealing, the annealed portion is stable as it is longer, but in the case of a short probe to be used for detection of genetic disease, etc., the ratio of the annealed portion based on the whole is increased and the labelled portion is decreased, whereby specific activity as the probe is lowered.

On the other hand, in the hybridization reaction, a labelled oligonucleotide or polynucleotide, namely a probe can form a base pair with the target nucleic acid.

There has been known a method in which a hybrid is formed with the target nucleic acid after fixing on a carrier having the function capable of separating the probe nucleic acid from the unreacted material, and then the hybrid is separated by the centrifugation method. As an example, there is Japanese Patent Application Laid-Open No. 63-117262, etc. More specifically, according to these methods, first a nucleic acid under the state of a single strand containing the base sequence complementary to a specific base sequence of the target is bound to the surface of particles comprising an organic polymer substance with nonporous surfaces having particle sizes of 0.01 to 50 μm , to form a probe nucleic acid for formation of hybrid bound to a carrier having the function capable of being separated from the unreacted material. Next, the probe nucleic acid is reacted with the sample to form a hybrid with the target nucleic acid containing the specific base sequence. By eliminating the nucleic acid having formed no hybrid with the probe nucleic acid on the carrier and other intervening matters by the centrifugation method, etc., the target nucleic acid forming the hybrid is detected. Otherwise, as the method for separating or fixing the material to be detected, there have been known the following two methods.

One method is (1) to modify chemically the 5'- or 3'-end of a probe nucleic acid, and a specific example is to bind a substance for capturing an insoluble carrier to the 3'-end side of the probe nucleic acid by use of Terminal Deoxynucleotidyl Transferase as shown in Japanese Patent Application Laid-Open No. 63-243875.

The second method is (2) to fix a nucleic acid as a whole by use of an organic solvent. This is described in detail in, for example, Japanese Patent Application Laid-Open No. 59-122499.

In the hybridization reaction, first of all, the reaction is carried out at a constant temperature so that the probe and the target DNA may form a hybrid through hydrogen bonds. Here, if the temperature is too high, the probe and the DNA having the complementary sequence cannot be bonded, while if it is too low, the probe will be bound non-specifically to the DNA. Also, if formamide is added into the reaction mixture, interference with formation of nonspecific hybrid occurs.

Next, after the hybridization reaction, by lowering the salt concentration in the solution or elevating the temperature of the solution, the probe non-specifically bound is washed away. By these operations, only the DNA fragment having the sequence complementary to the probe can be detected.

In the case of a long probe (1000 base pairs), these conditions are substantially established, but setting of conditions in the case of using a synthetic oligonucleotide is considerably difficult. Presently, those of 30 mers or less are frequently utilized as the oligonucleotide probe, but in the case of such short probes, the reaction conditions of hybridization are made moderate so that it may surely form the hybrid with the desired DNA. In other words, setting of conditions such as lowering of temperature or reducing the content of formaldehyde, etc. are required. Also, for washing after the reaction, the conditions must be selected for eliminating the nonspecific bonds of the probe without destroying hybrid formation in itself, but trials and errors are necessary for setting of such conditions.

For solving this problem, there has been frequently employed a method in which two kinds of oligonucleotide probes are prepared, screening is performed with the first probe, and thereafter screening is performed again with the second probe. However, this method requires much care and is time consuming.

Rather than such method, it is preferable to use a longer probe and remove completely the nonspecific hybrids by making the conditions of hybridization and the conditions of washing more strict.

In other words, as the length of the DNA oligonucleotide to be utilized as the capturing probe is longer, an error in complementary binding during hybridization can be prevented. However, in the two methods of the prior art (the methods (1) and (2) as described above), the necessary DNA as a whole is required to be synthesized in both cases.

On the other hand, there is the problem at the synthesis yield by means of a DNA synthesizer is lowered as the oligonucleotide to be synthesized is longer. In addition, the method of bonding by application of chemical modification at the 5'- or 3'-end has the following problems.

Since the site to be modified is limited to about 1 or 2 places, sufficient fixation can be obtained with difficulty, and it is difficult to obtain a probe with high capturing ability.

Also, in the case of a long nucleic acid for capturing, the probability of existence of the complementary sequence in the nucleic acid is enhanced, and it forms itself a higher structure during the hybridization reaction, playing no longer the function of capturing, whereby the effect of hybrid formation is markedly reduced.

In the method of carrying the nucleic acid as a whole by use of an organic solvent, although it can be avoided that the nucleic acid for capturing itself takes a higher structure as described above, it will take unpractically a long time for searching for the conditions of fixing the nucleic acid without changing the characteristics of the nucleic acid as a whole.

In "Nature" 320, April 24, 1986, 695-699, a method is suggested for deducing the amino-acid sequence of the precursor of the human tumor cell line-derived platelet-derived growth factor A-chain from complementary DNA clones.

"Nucleic Acids Research" 13, 5, 1985, 1529 to 1541 discloses a method for chemical labelling of DNA probes with biotin at the 5'-terminus. That is, according to this reference, the labelling substance is connected to a terminal of the single-stranded oligonucleotide only.

EP-A-0 168 342 is directed to a method for the preparation of a DNA sequence coding for the amino-acid sequence of Hirudine. According to this reference radio-active labelling is carried out on the 5'-end of the obtained fragments.

There is no indication as to introducing a fixing substance into the probe oligonucleotides during the preparation of the probe oligonucleotide in the extension reaction.

In "Nature" 318, November 28, 1985, 334-338, a method is disclosed for the protein sequencing of the signal recognition particle receptor (SRP) from the complementary DNA sequence. According to this method obtained single cDNA-strands were bonded together and the complementary sequences were filled by using radio-active labelled nucleotides.

"The Journal of Biological Chemistry" 257, 16, August 25, 1982, 9226-9229, is directed to a method for the synthesis of double-stranded DNA segments. The fragments are annealed at their 3'-ends. The thus obtained double-stranded products were polymerized by the addition of the four deoxyribonucleoside triphosphates.

The DNA sequences were determined by labelling of restriction fragments at their 3'-ends with radio-active dCTP. However, there is no indication as to introducing a fixing substance, nor to the introduction of such substances at positions other than the ends.

According to EP-A-0 196 762 the coding sequence for Ricin B was cloned. Further, a novel means for reconstructing missing portions of coding sequences is disclosed. Again, the single-stranded DNA is labelled at one end only by a radio-active marker.

SUMMARY OF THE INVENTION

Accordingly, an object of the present invention is to provide methods for simple detection and reparation of a target oligonucleotide with good precision as well as high synthesis yield.

Another object of the present invention is to provide a method for forming a labelled double-stranded oligonucleotide having any desired length with good precision and high synthesis yield, and which can further form a labelled double-stranded oligonucleotide functioning as a probe with high specific activity.

Also, still another object of the present invention is to provide a method for preparing an oligonucleotide to be used as the probe for capturing which solves of such drawbacks of the prior art and said oligonucleotide.

In short, the present invention provides a method for separating a target oligonucleotide, comprising :

a) preparing a probe oligonucleotide having a fixing substance by the steps of :

- i) synthesizing at least one pair of oligonucleotides capable of binding with each other through complementary sequences at the 3' - end regions of the oligonucleotides,
- ii) binding the at least one pair of oligonucleotides through the complementary sequences to form partially double-stranded oligonucleotide, and then carrying out an extension reaction in a reaction solution containing a nucleotide to which a fixing substance is bound to polymerise nucleotides onto the partially double-stranded oligonucleotide and to incorporate the fixing substance into the extended part of the double-stranded oligonucleotide obtained by the extension reaction,
- iii) denaturing the double-stranded oligonucleotide in which the fixing substance is incorporated to form single-stranded oligonucleotide having the fixing substance ;

b) reacting the single-stranded oligonucleotide having the fixing substance with a target single-stranded oligonucleotide in a mixture of different oligonucleotide to obtain a double-stranded hybrid ;

c) binding the incorporated fixing substance to a carrier which is specifically reactive with the fixing substance ; and

d) separating the hybrid bound to the carrier from the mixture.

There is also provided a method for detecting a target oligonucleotide comprising:

a) preparing a probe oligonucleotide having a fixing substance by the steps of :

- i) synthesizing at least one pair of oligonucleotides capable of binding with each other through complementary sequences at the 3'-end regions of the oligonucleotides,
- ii) binding the at least one pair of oligonucleotides through the complementary sequences to form partially double-stranded oligonucleotide, and then carrying out an extension reaction in a reaction solution containing a nucleotide to which a fixing substance is bound to polymerise nucleotides onto the partially double-stranded oligonucleotide and to incorporate the fixing substance into the extended part of the double-stranded oligonucleotide obtained by the extension reaction,
- iii) denaturing the double-stranded oligonucleotide in which the fixing substance is incorporated to form single-

stranded oligonucleotide having the fixing substance ;

b) reacting the single-stranded oligonucleotide having the fixing substance with a target single-stranded oligonucleotide in which a labelling substance is contained to obtain a double-stranded hybrid containing the labelling substance ;

c) binding the incorporated fixing substance to a carrier which is specifically reactive with the fixing substance ;

d) separating the double-stranded hybrid bound to the carrier ; and

e) detecting the labelling substance in the separated double-stranded hybrid.

There is also provided a method for separating a target oligonucleotide, comprising :

a) preparing a probe oligonucleotide having a fixing substance by the steps of :

i) synthesizing first and second oligonucleotides capable of binding with each other through complementary sequences at the 3'-end regions of the oligonucleotides, and synthesizing third oligonucleotide capable of binding with either of said first or second oligonucleotide through complementary sequences at the 5'-end region of the oligonucleotides,

ii) binding the first, second and third oligonucleotides through the complementary sequences at the 3'-end and 5'-end regions of the oligonucleotides to form partially double-stranded oligonucleotide, and then carrying out an extension reaction in a reaction solution containing a nucleotide to which a fixing substance is bound to polymerise nucleotides onto the partially double-stranded oligonucleotide and to incorporate the fixing substance into the extended part of the double-stranded oligonucleotide obtained by the extension reaction,

iii) denaturing the double-stranded oligonucleotide in which the fixing substance is incorporated to form single-stranded oligonucleotide having the fixing substance ;

b) reacting the single-stranded oligonucleotide having the fixing substance with a target single-stranded oligonucleotide in a mixture of different oligonucleotides to obtain a double-stranded hybrid ;

c) binding the incorporated fixing substance to a carrier which is specifically reactive with the fixing substance ; and

d) separating the double-stranded hybrid bound to the carrier from the mixture.

There is also provided a method for detecting a target oligonucleotide, comprising :

a) preparing a probe oligonucleotide having a fixing substance by the steps of :

i) synthesizing first and second oligonucleotides capable of binding with each other through complementary sequences at the 3'-end regions of the oligonucleotides, and synthesizing third oligonucleotide capable of binding with either of said first or second oligonucleotide through complementary sequences at the 5' - end region of the oligonucleotides,

ii) binding the first, second and third oligonucleotides through the complementary sequences at the 3'-end and 5'-end regions of the oligonucleotides to form partially double-stranded oligonucleotide, and then carrying out an extension reaction in a reaction solution containing a nucleotide to which a fixing substance is bound to polymerise nucleotides onto the partially double-stranded oligonucleotide and to incorporate the fixing substance into the extended part of the double-stranded oligonucleotide obtained by the extension reaction,

iii) denaturing the double-stranded oligonucleotide in which the fixing substance is incorporated to form single-stranded oligonucleotide having the fixing substance ;

b) reacting the single-stranded oligonucleotide having the fixing substance with a target single-stranded oligonucleotide to which labelling substance is incorporated in a mixture of different oligonucleotide to obtain a double-stranded hybrid containing the labelling substance ;

c) binding the incorporated fixing substance to a carrier which is specifically reactive with the fixing substance ;

d) separating the double-stranded hybrid bound to the carrier from the mixture; and

e) detecting the labelling substance in the separated double-stranded hybrid.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1, Fig. 2 and Fig. 3 are schematic diagrams for illustration of the partial double strands to be used in the present invention; Fig. 4 is a diagram for illustration of Example 2 and Example 8; and Fig. 5 is the illustration showing the results of electrophoresis for the product of Example 3.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention has been achieved so that each chain of the double strand may function as both of a template and a primer for oligonucleotide polymerization by improvement of the primer extension method of the prior art.

The template and the primer in the present invention, when two kinds of oligonucleotides synthesized form a partial double strand with complementary sequences, refer to the protrudent single strand which is the non-binding portion as the template, while of the respective chains constituting the partial double strand, the chain participating in binding on the opposite side to the protrudent single strand is referred to as the primer.

In short, the method for forming a double-stranded oligonucleotide used in the present invention may be specifically described as follows.

(a) Two single-stranded oligonucleotides with complementary sequences at the 3'-ends are synthesized by a DNA synthesizer. (b) Next, these two oligonucleotides are bound at the complementary sequences by the annealing reaction to form partially a double strand. (c) The partial double strand formed at this time functions as the primer of the DNA's synthesized respectively. With different nucleotide triphosphates and reagents for polymerization of the nucleotide triphosphates, a double-strand is formed while synthesizing oligonucleotides having complementary sequences in the direction from the 5'-side to the 3'-side with the protrudent single strand as the template.

To describe in more detail, the length of the oligonucleotide synthesized in (a) may be shorter than that practically required. The total hydrogen bond number in the complementary region when a pair of oligonucleotides are formed may be preferably 16 to 24, but may be shorter than that, provided that it is 13 or more, more preferably 16 or more, or may be even longer.

(b) In the annealing reaction (binding step), the mixture of the two kinds of single-stranded oligonucleotides is heated in an appropriate buffer at 65 °C or higher for one minute or longer, preferably at 65 °C for 10 minutes, or at 95 °C for one minute or longer, and then the solution is left to cool to room temperature. According to this reaction, the oligonucleotides are bound at the sequences complementary to each other to form a partial double-strand.

(c) Into the solution are added appropriate amounts of dATP, dCTP, dGTP, and TTP as synthetic reagents which are nucleotide triphosphates.

Also, for the enzyme to be used as the polymerizing reagent, there may be employed *E. coli* DNA polymerase I, Klenow fragment of DNA polymerase, T4DNA polymerase (T. Maniatis et al, Molecular Cloning 108, Cold Spring Harbor Laboratory), T7DNA polymerase (S. Tabor et al, Proc. Natl. Acad. Sci. USA, 84, 4767-4771 (1987), thermally stable DNA polymerase (R.k. Saiki et al, Science, 239, 487-491 (1988), other available DNA polymerases, reverse transcriptases, and other enzymes, such as enzymes promoting binding of nucleotides in appropriate modes for forming the primer extension products of the respective nucleic acids which are complementary.

The polymerization reaction may be performed at the temperature where any of the above-mentioned polymerizing reagent can function without cleavage of the hydrogen bonds at the annealed portion. For example, a system wherein polymerization reaction is carried out at 0 to 40 °C by use of Klenow fragment of DNA polymerase is preferred.

As described above, a double-stranded oligonucleotide is formed via a partial double-stranded oligonucleotide.

By forming a double-stranded oligonucleotide according to the formation method as described above, a double-stranded oligonucleotide comprising oligonucleotides with a length of 100 mer or more can be simply prepared.

1) According to the primer extension method of the prior art, it has been difficult to prepare a nucleotide oligomer of about 100 mer at once with good precision and with good yield. This is because, for the purpose, synthesis of a template DNA having such length is required, and also the synthesis of the DNA synthesizer is limited to about 100 mer. Besides, when 100 mer is attempted to be synthesized, the synthesis yield is 10 % or lower under the present situation. Therefore, only those with desired lengths are required to be purified to remove by-products before the next reaction. However, it is very difficult to purify with good precision without an error of single base.

In contrast, in the method of the present invention, it is satisfactory to synthesize oligonucleotides with lengths shorter than the length of the probe. Accordingly, when a gene of 100 base pairs is to be prepared, if the annealing portion is made 8 base pairs, for example, two oligonucleotides with a length of 54 bases may be synthesized. As a matter of course, as compared with the case of synthesizing an oligonucleotide with 100 bases, the case of 54 bases has better synthesis efficiency and also less by-products. Therefore, purification is also simple.

Further, in the method according to the present invention, it becomes possible to synthesize a gene of 100 mer or

more, for example, to synthesize a gene of 200 base pairs, etc. at once, which has been almost impossible in the prior art. By synthesizing two oligonucleotides of 100 mer where 100 mer is the limit that can be synthesized at once in a synthesizer, and performing the extension reaction with enzymes, a gene with a length approximate to 200 base pairs can be synthesized.

2) Also in the reaction with polymerization reagents, in the case of the prior art method, a large number of nucleotides must be polymerized with enzymes. Whereas, the lengths which can be extended with enzymes are also limited. As the result, the reaction is stopped in the course, whereby shorter genes are easily formed.

In the case of the present invention, the length of the nucleotide polymerized with an enzyme is considerably shorter than in the prior art. In the case of preparing a gene of 100 mer, 92 bases are extended in the prior art, while in the present invention 46 bases are satisfactory to be extended. As compared with extension of 92 bases, the case of extending 46 bases can proceed within a short time and more completely. For this reason, a double-stranded DNA with uniform lengths can be obtained, whereby ligation reaction efficiency to vector, and transformation efficiency are improved to a greater extent.

A further method for obtaining a double-stranded probe oligonucleotide used in the present invention has been achieved so that each chain of a plurality of oligonucleotides may function as both of a template and a primer by improvement of the primer extension method of the prior art.

The template and the primer in this method, when plural kinds of oligonucleotides synthesized form a partial double strand with complementary sequences, refer to the protrudent single strand which is the non-binding portion as the template, while of the respective chains constituting the partial double strand, the chain participating in bonding on the opposite side to the protrudent single strand is referred to as the primer.

In short, this method may be specifically described as follows.

For a desired nucleic acid base sequence (oligonucleotide), (1) as shown in Fig. 1, a plural number of single-stranded oligonucleotides having complementary sequences at the 3'-end or the 5'-end are synthesized by a DNA synthesizer. (2) Next, phosphoric acid groups are attached at the 5'-ends of the respective oligonucleotides. (3) Next, these two oligonucleotides are bound at the complementary sequences by the annealing reaction to form partially a double strand. (4) One of the partial double strands formed here functions as the primer for the other oligonucleotide synthesized. With different nucleotide triphosphates and reagents for polymerization of the nucleotide triphosphates, a double strand is formed while synthesizing oligonucleotides having complementary sequences in the direction from the 5'-side to the 3'-side with the protruded single strand portion as the template. (5) When there is a nick formed in the oligonucleotide thus prepared, ligation may be performed with an enzyme so that there may be no nick.

To describe in more detail, the length of the oligonucleotide synthesized in (1) is the length which can be synthesized at once in a DNA synthesizer (generally 100 mer or less). The total hydrogen bond number in the complementary region when a pair of oligonucleotides are formed may be preferably 16 to 24, but may be shorter than that, provided that it is 13 or more, more preferably 16 or more, or may be even longer.

(2) The method for attaching phosphoric acid group to the 5'-end may be either by way of chemical synthesis or by use of an enzyme such as T4 polynucleotide kinase, etc. in the presence of ATP.

At least by attachment of phosphoric acid at the end of the oligonucleotide according to the operation, when the nucleic acid bases are polymerized in the above step (4), depending on selection of the polymerization reagent, ligation may be sometimes performed between the nucleic acid base naturally polymerized during polymerization and the end portion of the oligonucleotide attached with phosphoric acid group. However-, when ligation is insufficient with such operation, ligation is performed by use of a ligating reagent as described below in (5).

(3) In the annealing reaction, the mixture of the plural kinds of single-stranded oligonucleotides is heated in an appropriate buffer at 65 °C or higher for one minute or longer, preferably at 65 °C for 10 minutes, or at 95 °C for one minute or longer, and then the solution is left to cool to room temperature. According to this reaction, the oligonucleotides are bound at the sequences complementary to each other to form a partial double strand.

(4) Into the solution are added appropriate amounts of dATP, dCTP, dGTP, and TTP as synthetic reagents, which are nucleotide triphosphates.

Also, for the enzyme to be used as the polymerizing reagent, E. coli DNA polymerase I, Klenow fragment of DNA polymerase, T4DNA polymerase (T. Maniatis et al, Molecular Cloning 108, Cold Spring Harbor Laboratory), T7DNA polymerase (S. Tabor et al, Proc. Natl. Acad. Sci. USA, 84, 4767-4771 (1987), thermally stable DNA polymerase (R. k. Saiki et al, Science, 239, 487-491 (1988), other available DNA polymerases, reverse transcriptases, and other enzymes, such as enzymes promoting binding of nucleotides in appropriate modes for forming the primer extension products of the respective nucleic acids which are complementary.

The polymerization reaction may be performed at the a temperature where any of the above-mentioned polymerizing reagent can function without cleavage of the hydrogen bonds at the annealed portion. For example, a preferred system is such that polymerization reaction is carried out at 0 to 40 °C by use of Klenow fragment of DNA polymerase.

The reaction of (5) is carried out with a DNA ligase. DNA ligase may be either one derived from E. coli or one derived from phage T4, or alternatively another enzyme which can function similarly.

As described above, a double-stranded oligonucleotide is formed via a partial double-stranded oligonucleotide.

In the case of preparing a long DNA by successive ligation of double-strands successively synthesized, for identification of the directionality during ligation, purifications in the respective ligation steps are required. Then the final yield will be lowered.

In contrast, in the formation method used in the present invention as described above, the chain length is extended in the extension reaction after formation of the hydrogen bonds previously at the complementary sequences. There is the advantage that the reaction can be carried out all at once by use of a mixture of all the oligonucleotides.

Further, the method is improved in yield to great precision without purification.

When a gene of 500 base pairs is to be prepared by use of the formation method used in the present invention (that is, when the portion of annealing is made 8 base pairs, for example, a double-stranded oligonucleotide is formed by synthesizing 5 oligonucleotides with a length of 100 mer and one oligonucleotide of 40 mer), as compared with 10 oligonucleotides of the prior art, the number of oligonucleotides can be half. Then reagents and time can be saved.

Further, as compared with the prior art, treatments in successive ligations and checking at the respective steps can be omitted.

Also, when a double-stranded oligonucleotide with a length of 100 base pairs is attempted to be formed according to the successive ligation method, preparing 4 oligonucleotides of 50 mer, there is the problem of low yield because of the directionality undetermined during ligation. Accordingly, it may be preferable to use a method to prepare two oligonucleotides of 100 mer at once, and form a double strand by annealing.

However, the method has also the following problem. When an oligonucleotide of 100 mer is tried to be synthesized at once, the synthesis yield is 10 % or less under the present situation. Accordingly, it is necessary to purify only those with desired lengths to remove by-products before the next reaction, but it is very difficult to purify the product with good precision without an error of single base.

In contrast, by use of the above method, a double-stranded oligonucleotide can be synthesized by ligating the oligonucleotides with shorter lengths without the problem of low yield, etc. as explained in the successive ligation method as described above. For example, a double-stranded oligonucleotide with a length of 100 base pairs is formed according to the above method, for example, 4 oligonucleotides with a length of 25 mer and one oligonucleotide with a length of 24 mer may be synthesized by designing the annealing portion of 6 base pairs.

In short, it can not only overcome a problem of directionality during ligation in the successive ligation method as described above, but also synthesis efficiency is better in synthesis of an oligonucleotide of 25 mer than in synthesis of an oligonucleotide of 100 mer, with the by-products being also less. Therefore, purification becomes also simpler.

Further, in the case where the double-stranded oligonucleotide is labelled, there are the following advantages by use of the above method.

In the nick translation method of the prior art, because the position where the site of incorporated nick could not be identified, the labelling position and labelling amount could not be controlled. Accordingly, the strength of the probe differed depending on the preparation conditions, and therefore it was difficult to prepare always a probe having a constant strength.

In the case of the above methods, during probe designing, the labelled amount can be previously predicted, and the probe can be prepared while controlling it according to the purpose.

Further, it is also possible to prepare a DNA having alternately labelled regions and non-labelled regions by adding only a labelling substance during the enzyme reaction.

The oligonucleotide double strand thus prepared has the labelling substance incorporated in both the chains, each chain functioning as a probe having high specific activity.

In the primer extension method of the prior art, labels could be incorporated into those with a length of 50 base pairs or less, while in the nick translation method, into those with a length of 500 base pairs or more. In the present invention, it is also possible to prepare a probe with an intermediate length between these.

On the other hand a double stranded oligonucleotide having a fixing substance is formed by utilizing the methods described above and as described similarly below.

More specifically, (d) two or more of single-stranded oligonucleotides having 6 bases pairs or more through complementary sequences at the 3'-ends are synthesized by a DNA synthesizer. (e) Next, these at least two oligonucleotides are bound at the complementary portions according to the annealing reaction to constitute partially a double strand. (f) The partial double strand formed at this time functions as the primer for the two DNA's (oligonucleotides) respectively synthesized. With different nucleotides triphosphate and a nucleotide triphosphate derivative which is a fixing substance, and reagents for polymerization of the nucleotide triphosphates and nucleotide triphosphate derivative, and with the protrudent single strand portion as the template, a double strand is synthesized while synthesizing an oligonucleotide having complementary sequence in the direction from the 5'-side to the 3'-side. In the case, the fixing substance is introduced into either one or both of a pair of oligonucleotides, but an oligonucleotide with higher yield can be formed in the latter, because one having the fixing substance introduced into both can readily react with a carrier. Further, in the present invention, (g) the double-stranded oligonucleotide obtained in the step is separated

(g) into single strands single-stranded oligonucleotides. The step (f) is described in more detail.

At this time, one or more kinds of fixing substance are added.

The fixing substance as mentioned in the present invention is a nucleotide triphosphate derivative having a binding group which is bound to the nucleotide triphosphate introduced into the DNA hybrid forming body by the extension reaction by use in the polymerizing reagent in combination through the specific affinity with a carrier. Specifically, as the above-mentioned nucleotide triphosphate derivative, biotin, heavy metal derivatives, homopolynucleotides, etc. may be employed.

Also, as the enzyme to be used as the polymerizing reagent, there may be included *E. coli* DNA polymerase I, Klenow fragment of DNA polymerase, T4DNA polymerase (T. Maniatis et al, Molecular Cloning 108, Cold Spring Harbor Laboratory), T7DNA polymerase (S. Tabor et al, Proc. Natl. Acad. Sci. USA, 84, 4767-4771 (1987)), thermally stable DNA polymerase (R.k. Saiki et al, Science, 239, 487-491 (1988)), other available DNA polymerases, reverse transcriptases, and other enzymes, such as enzymes promoting binding of nucleotides in appropriate modes for forming the primer extension products of the respective nucleic acids (oligonucleotides) which are complementary.

By forming a double-stranded oligonucleotide according to the above-described formation method, a double-stranded oligonucleotide comprising oligonucleotides with a length of 100 mer will be simply prepared.

By separating the double-stranded oligonucleotide into single strands, an oligonucleotide having a fixing substance introduced therein can be prepared. By hybridizing the oligonucleotide having the fixing substance introduced therein as the probe oligonucleotide (nucleic acid) with a target oligonucleotide (sample nucleic acid), through the above-mentioned fixing substance, the hybrid and the carrier are bound, whereby said hybrid forming body can be separated with good efficiency from the non-hybrid forming body.

The affinity pair portion formed between the fixing substance and the carrier as mentioned above is a component having affinity for other components. For example, there may be included antigen-antibody such as biotin-avidin or streptoavidin, hapten-antibody, flavin adenine nucleotide (FAD)-glucose oxidase, biotin-antibiotin antibody, etc., heavy metal derivative-thio group and various homopolynucleotides such as polydG-polydC, polydA-polydT and polydA-polyU as such affinity pairs.

The above carrier is bound to the nucleotide triphosphate derivative as mentioned above through affinity, but not to nucleotide triphosphate, and has the property capable of separating the hybrid forming body from the non-hybrid binding body by binding to the nucleotide triphosphate derivative.

As the separation method, there are methods such as centrifugation, adsorption, etc.

To describe in more detail about separation, the separation method and a suitable kind of carrier can be selected depending on whether the procedure for binding the carrier to the fixing substance is before or after formation of the hybrid forming body. For example, after hybridization of the probe having the fixing substance introduced therein with the target nucleic acid, the above-mentioned carrier is bound to the fixing substance, and the bound product can be separated by centrifugation or by having the carrier onto the fixing substance. Alternatively, a probe already having separation function may be formed by having an insoluble carrier having the function of being separated bound to the fixing substance before hybridization, and then the probe may be hybridized with the target nucleic acid and separated by centrifugation. The carrier is selected particularly from the following substance (hereinafter called ligand) is selected. Here the ligand has the property capable of bonding to both the fixing substance and the insoluble carrier.

Examples of the ligand may include antibodies such as streptoavidin, avidin, glucose oxidase, antibiotin antibody, etc. And, by bonding the fixing substance to the ligand, and further bonding the ligand to the insoluble carrier, a probe to be separable is prepared.

As the organic polymer substance to be used for the insoluble carrier to be separable by centrifugation, there can be included water-insoluble organic polymer substances obtained by polymerization of at least one of vinyl type monomers comprising aromatic vinyl compounds, esters or amides of unsaturated carboxylic acids, unsaturated nitriles, halogenated vinyl compounds, conjugated diene compounds, and lower aliphatic vinyl esters, water-insoluble polymer substances obtained by chemical modification of said organic polymer substances, or crosslinked products of polysaccharides such as agarose, dextran, cellulose, etc., and crosslinked products of methylated albumin, gelatin, collagen, casein, etc.

The oligonucleotide of the present invention can be said to be a probe for capturing prepared by a simple method.

Furthermore an oligonucleotide obtained by another method is to be described below.

For a desired nucleic base sequence, (6) a plural number of single-stranded oligonucleotides having complementary sequences of 6 base pairs or more at the 3'-end or the 5'-end are synthesized by a DNA synthesizer as shown in Fig. 1. (7) Next, phosphoric acid groups are attached to the 5'-ends of the respective oligonucleotides. (8) Next, these two oligonucleotides are bound at the complementary portions according to the annealing reaction to constitute partially a double strand. (12) One of the partial double strands formed at this time functions as the primer for the other oligonucleotide synthesized. With various different nucleotide triphosphates and a nucleotide triphosphate which is the fixing substance, and reagents for polymerization of the nucleotide triphosphates and nucleotide triphosphate derivative, and with the protrudent single strand as the template, a double strand is synthesized while synthesizing an oligonucleotide

having complementary sequence in the direction from the 5'-side to the 3'-side. (10) When a nick is formed in the oligonucleotide thus prepared, ligation may be performed with an enzyme so that there may be no nick. (11) The double strand is separated to form single-stranded oligonucleotides.

The step (12) is to be described in more detail.

(12) Into the solution are added appropriate amounts of dATP, dCTP, dGTP and TTP as synthetic reagents which are nucleotide triphosphates.

At this time, one or more kinds of fixing substances is added. The fixing substance and the enzyme to be used as the polymerizing reagent are already described in the above.

When the oligonucleotide nucleic acid sequence is correlated with genetic diseases, tumor diseases or infectious diseases, it can be effectively utilized for analysis.

A feature of the above method resides in confirming that, in the respective formation method of oligonucleotides as described, more preferably, the complementary base sequence portion has 13 or more of hydrogen bonds formed between one oligonucleotide and the other oligonucleotide.

The present invention is described below in more detail by referring to Examples.

Reference Example 1

Two kinds of oligonucleotides as shown below each having EcoRI site at the 5'-end were synthesized by means of a DNA synthesizer (Model 381A, available from Applied Biosystems).

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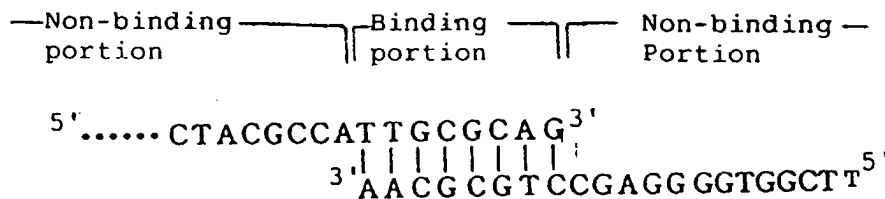
5' GCGCTAATGGGAATTCGGACGCTCTATTTC
CTCGTGAAAGGGATGGGCGTTGCGGACCCAGATGC
AAAGAAATTCTACGCCATTGCGCAG
5' CTCGCCCAGGAATTCCGTTACGATGTACC
TCTCGATGCTGCTGGGGTATGGCCTCACAATGGTAC
CGTTCGGTGGGGAGCCTGCTGCGCAA

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Eight bases from the 3'-end are complementary to each other.

For a part of these oligonucleotides synthesized, 10 % polyacrylamide gel (containing 7 M urea) electrophoresis was conducted for examination of its purity. As the result, the purity was found to be about 10 %, and therefore each 2 µg oligonucleotide was purified according to the above electrophoresis, extracted with 1 mM EDTA to be used in the following reactions.

Each oligonucleotide in an amount of 2 µg was placed in an Eppendorf tube, 5 µl of a 10 x annealing solution (100 mM Tris-HCl, pH 8.0; 60 mM, MgCl₂; 60 mM β-mercaptoethanol; 500 mM NaCl) was added, and the mixture was made up to 50 l with distilled water. The mixture was heated in a beaker containing hot water of 65 °C for 10 minutes, then cooled slowly to room temperature (time required was about one hour). According to this reaction, the two oligonucleotides form a partial double strand as shown below.



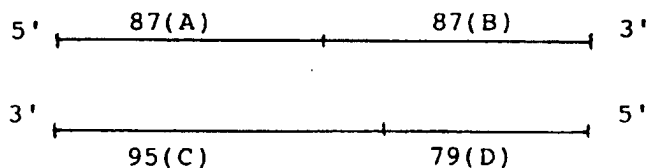
Into 50 µl of this solution were added each 2 µl of 1 mM dATP, dGTP, TTP, and dCTP, 5 µl of 10 x annealing solution, 32 µl of distilled water, and after thorough mixing, 16 units of Klenow fragment of DNA polymerase (TOYOBO) were added, followed by heating at 37 °C for one hour to perform the extension reaction.

Then, for confirming whether the double-stranded DNA has been prepared as programmed, after phenol extraction, ethanol precipitation were performed from the above reaction solution, digestion was performed with a restriction en-

zyme EcoRI, and the resultant fragment was inserted at the EcoRI site of a cloning vector pUC 19. When *E. coli* JM 109 was transformed with the plasmid pUC19 having the synthesis gene inserted therein, colonies bearing the plasmid containing the double-stranded gene with high efficiency (10^6 colonies or more per 1 μ g of DNA) were obtained. In view of the fact that no ligation is effected to the EcoRI site of pUC19 when synthesis is incomplete, it can be said that the extension reaction with the enzyme has been completely performed.

From the colonies, DNA was prepared according to the quantitation method (the DNA sequence determination method with modified T7DNA polymerase; Saibo Kogaku (Cell Engineering) vol. 7, No. 9, 1988, p. 61), and the sequence was confirmed by use of DNA SEQUENCING SYSTEM (370A, ABI). As the result, it was confirmed that the double-stranded DNA was synthesized exactly as initially designed.

Next, comparison was made with the prior art examples. A DNA of 174 base pairs similar to the above desired DNA was prepared all by synthesis according to the prior art method. Four oligonucleotides having base lengths of 87, 87, 95 and 79 as shown below were synthesized.



Since the yield of each oligonucleotide was about 10 %, each oligonucleotide was purified by electrophoresis, and the band corresponding to each length was cut out from the gel and the desired oligonucleotide was extracted from the band. When the extract was examined newly by gel electrophoresis, each oligonucleotide was found to contain 10 % or more of an oligonucleotide having shorter length by one or more base (as the result of intermission of synthesis) mixed therein.

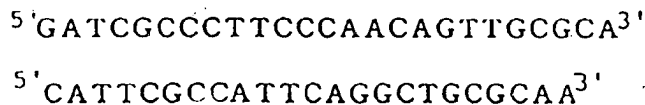
These four oligonucleotides were ligated, digested with a restriction enzyme, ECORI, and the fragment was inserted at the EcoRI site of a cloning vector pUC19. When *E. coli* JM109 was transformed with the plasmid pUC19 having the synthetic gene inserted therein, the transformation efficiency was found to be about 10^5 colonies per 1 μ g of DNA.

Such lowering in transformation efficiency may be considered to be due to the lowered ligation efficiency by mixing of the oligonucleotide shorter by one or more base during ligation of the two oligonucleotides (A and B or C and D) in each chain.

This Example is effective in that not only the labors for synthesis are half of the prior art example, but also such lowering in efficiency during ligation can be avoided.

Reference Example 2

Two kinds of oligonucleotides as shown below corresponding a part of the sequence of the plasmid pUC19 were synthesized by means of a DNA synthesizer (Model 381A, Applied Biosystems).



Eight bases from the 3'-end are complementary to each other.

For a part of these oligonucleotides synthesized, 20 % polyacrylamide gel (containing 7 M urea) electrophoresis was conducted for examination of its purity. As the result, the purity was found to be about 95 % or more, and therefore each oligonucleotide was used in the following reactions without further purification.

Each oligonucleotide in an amount of 2 μ g (about 130 pmole) was placed in an Eppendorf tube, 5 μ l of a 10 x annealing solution (100 mM Tris-HCl, pH 8.0 ; 60 mM, MgCl₂ ; 60 mM β -mercaptoethanol; 500 mM NaCl) was added, and the mixture was made up to 50 μ l with distilled water. The mixture was heated in a beaker containing hot water of 65 $^{\circ}$ C for 10 minutes, then cooled slowly to room temperature (time required was about one hour). According to this reaction, the two oligonucleotides form a partial double-strand as shown below.

5' GATCGCCCTTCCCAACAGTTGCCGAG 3'

3' AACGCGTCGGACTTACCGCTTAC 5'

Into 50 μ l of this solution were added each 2 μ l of 1 mM dATP, dGTP, and dCTP, 5 μ l of 0.4 mM biotinylated UTP (BRL), 5 μ l of 10 x annealing solution, 32 μ l of distilled water, and after thorough mixing, 16 units of Klenow fragment of DNA polymerase (TOYOBO) were added, followed by heating at 37 °C for one hour to perform the extension reaction.

Then, for removing the unreacted biotinylated UTP, the reaction mixture was purified by a gel filtration column (Bio-gelp2; available from Bio Rad, 0.5 x 5 cm). The desired labelled nucleotide was recovered in the fractions which had passed through the column substantially as such.

Each 0.5 ml of the respective fractions was collected, and each 2 μ l was adsorbed on a nitrocellulose filter to perform color formation reaction of biotin according to the protocol of BRL. As the result, the second fraction formed the color strongly, whereby it was confirmed that the desired oligonucleotide was labelled with biotin.

Further, when a part of each fraction and the unreacted oligonucleotide were examined by Agarose gel electrophoresis, the oligonucleotide of the second fraction was clearly detected as one band longer than the unreacted oligonucleotide, whereby it was confirmed that the extension reaction with the enzyme was carried out completely.

When colony hybridization, Southern hybridization, and spot hybridization were performed with the use of this probe. Only the part of the pUC19 plasmid DNA formed the color clearly in all the cases.

Next, comparison was made with the prior art examples (the substitution synthetic method and the primer extension method).

A probe of 41 base pairs similar to the above desired probe was prepared according to the substitution synthetic method. That is, two oligonucleotides of 40 base length were synthesized, and after purification by gel electrophoresis (yield of about 50 %), formed into a double strand.

Next, the phosphoric acid at its 5'-end was removed, and in place thereof biotinylated dUTP was introduced with a polynucleokinase derived from T4 phage for labelling. The product was dissociated into single strands to be used as the probe.

Also, one of the above oligonucleotides of 41 base length and a primer of 8 base length complementary thereto at the 3'-end were synthesized and annealed, followed by the extension reaction with the Klenow fragment according to the same method as mentioned above to prepare a probe (the primer extension method).

5' GATCGCCCTTCCCAACAGTTGCCGAGCCTGAATGGCGAATG 3'
3' CCGCTTAC 5'

After denaturation of the plasmid DNA pUC19 and pBR322 with an alkali, DNA's in amounts of the group I No. 1 to 10, the group II No. 1 to 10 as shown in Fig. 4 was spotted on a nitrocellulose filter.

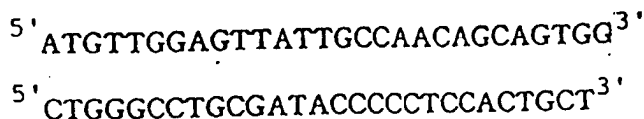
Three sheets of such filter were prepared, and after baking to 80 °C for 2 hours, hybridization reactions were carried out by use of three kinds of probes (two kinds of the prior art and the present invention in this Example 2).

When color formation reaction was carried out in conventional manner, in the probe according to the substitution synthetic method, color formation occurred under 5 ng at least in the group I, while no color formation observed in the Control of the group II. Next, in the case of using the probe according to the primer extension method, color formation occurred under up to 1 ng, and no color formation in the group II. In the probe prepared according to the method of the present invention, color formation occurred under 0.1 ng at least, but no color formation in the Control of the group II.

As the result of these experiments, it could be confirmed that not only the probe preparation is easier according to the method used for the present invention than according to the prior art methods, but also the detection sensitivity is elevated by 10 to 50-fold.

Reference Example 3

Bacteriorhodopsin which is a protein existing in *H. Halobium* was purified, and the amino acid sequence from its N-end was determined up to the 16th by a protein sequencer. Based on the result, the base sequence of the DNA was estimated, and the two oligonucleotides as shown below were synthesized by means of a DNA synthesizer.

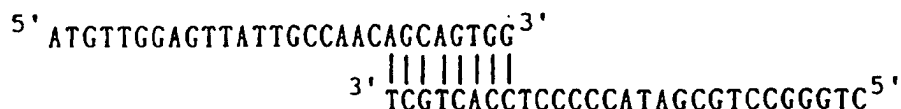


Eight bases from the 3'-end are complementary to each other.

For a part of these oligonucleotides synthesized, 15 % polyacrylamide gel (containing 7 M urea) electrophoresis was conducted for examination of its purity. As the result, the purity was found to be about 90 % or more, and therefore each oligonucleotide was used in the following reactions without further purification.

In an Eppendorf tube, to 45 ng (3 pmole) of each oligonucleotide was added 2 µl of a 10 x annealing solution (see Example 2) and the mixture was made up to 20 µl with distilled water.

The mixture was heated in a beaker containing 200 ml of hot water of 65 °C for 10 minutes, then cooled slowly to room temperature. According to this reaction, the two oligonucleotides were annealed as shown below.



Next, into 20 µl of the annealed oligonucleotide solution were added 2 µl of 10 x annealing solution, each 2 µl of 1 mM dATP, dCTP, and dGTP, further 100 µCi of ³²P-TTP, and further after making up the total liquid amount to 40 µl with addition of distilled water, one unit of Klenow fragment of DNA polymerase was added, followed by the reaction in ice for 1 hour.

For removing the unreacted ³²P-TTP from the reaction product, 20 µl of a 98 % formamide-0.05 % xylene cyanol-Bromophenol Blue solution was added into the reaction solution, heated at 90 °C for 2 minutes to denature, followed by 10 % polyacrylamide (containing 7 M urea) gel electrophoresis. After electrophoresis at 1000 V for 3 hours, the gel was removed from the glass plate, and left to stand for about 30 seconds with a X-ray film placed thereon. When this film was developed, a band as shown in Fig. 5 drawing was obtained. As estimated from the positions electrophoresed of xylene cyanol and Bromophenol Blue, the band corresponds to 48 mer, whereby it was confirmed that the synthetic oligonucleotide of 28 mer became the 48 mer according to the extension reaction. Besides, the extension reaction had been performed completely, and the band was single without observation of by-products of shorter lengths at all.

The gel corresponding to this position was cut out, and the desired oligonucleotides were extracted overnight at 37 °C with 1 mM EDTA solution, and its intensity was measured by a liquid scintillation counter to find that the probe prepared had a very strong intensity of 10⁸ cpm or higher.

The hybridization reaction by use of this probe gave good results in all of colony, Southern and spot hybridizations.

Reference Example 4

When the experiment was conducted in the same manner as in Reference Example 2 except for changing dATP, dGTP, dCTP and biotinylated UTP in Reference Example 2 to 2 µl of 1 mM ATP, GTP and CTP and 5 µl of 0.4 mM biotinylated UTP, respectively, good results were also obtained in said Reference Example 4.

Reference Example 5

The same experiment was conducted in the same manner as in Reference Example 2 except for adding further 2 µl of 1 mM TTP upon adding dATP, dGTP, dCTP. Example 5 also exhibited good results.

Reference Example 6

Three oligonucleotides as shown below were synthesized by means of a DNA synthesizer (Model 381, Applied Biosystems).

1) 5' TCCG' AATT CGGTACTGTTGCATGT'
 5 TGGAGTTATTGCCAACAGCAGTGGAG'
 GGGGTATCGCAGGCCAGATCACCGG
 10 ACGTTCTGGCTAGCGCTTCGGTACG 3'

2) 5' GCGCTAATGGGACTCGGGACGCTC
 15 TATTTCTCTCGTGAAAGGGATGGGGCGT
 CTCGGACCCAGATGCAAAGAAATTCT
 20 ACGCCATCACGACGGACCGTACCGA 3'

3) 5' ATTAGCGCCTCGTCCCAGCCATCG
 25 CGTTCACGATCTACCTCTCGATGCTG
 CTGGGGGTATGGCCTCACAAATGGTACC
 30 GTTCGTCGTAGAGTTAAACAACAG 3'

In the first and third synthetic oligonucleotides, the restriction enzyme EcoRI site is incorporated. Also, 8 base pairs from the 3'-end or the 5'-end (underlined portions) are complementary to each other.

A part of the oligonucleotide synthesized was examined for its purity by 10 % polyacrylamide gel electrophoresis containing 7 M urea to be judged as having a purity of about 10 %, and therefore a band with desired length was cut out from the gel, and the desired oligonucleotides were extracted with 1 mM EDTA, recovered by ethanol precipitation and then used in the following reactions.

Each oligonucleotide in an amount of 0.1 µg was placed in an Eppendorf tube, 10 µl of 10 x kinase buffer (0.5M Tris-HCl pH 8.0 ; 0.1M MgCl₂ ; 0.1M β-mercaptoethanol), 10 µl of 1 mM ATP and 1 µl of T4 polynucleotide kinase (10 units) (TOYOBO) were added, and the mixture was heated at 37 °C for one hour.

Next, 5 µl of 10 x annealing solution (100 mM Tris-HCl pH 8.0 H⁺, 60 mM MgCl₂ H⁺, 60 mM β-mercaptoethanol H⁺, 500 mM NaCl) Was added, and the mixture was made up to 50 µl with distilled water.

These oligonucleotides were heated in a beaker containing 200 ml of hot water of 65 °C for 10 minutes, and then left to cool slowly to room temperature. By this reaction, the three oligonucleotides were annealed as shown in Fig. 2.

Next, into 50 µl of the annealed oligonucleotide solution were added 5 µl of 10 x annealing solution, each 2 µl of 1 mM dATP, dCTP, dGTP and TTP, and further distilled water to make the total liquid amount to 100 µl, and then 12 units of Klenow fragment of DNA polymerase I were added, followed by the reaction at 37 °C for one hour to perform the extension reaction.

Further, after phenol extraction were performed from the above reaction mixture, the DNA was recovered by ethanol precipitation and its length was examined by 10 % polyacrylamide gel electrophoresis. As the result, a band was detected at the position of about 300 base pairs. Next, a primer of 8 base length complementary to the third synthetic oligonucleotides at the 3'-end were synthesized and annealed, followed by the extension reaction with the Klenow fragment according to the same method as mentioned above. After the DNA was recovered, digestion was performed in conventional manner with the restriction enzyme EcoRI, and the synthetic gene was ligated to the EcoRI site of the M13 phage. *E. coli* JM109 was transformed with the ligated product to obtain plaques bearing the plasmid containing the synthetic gene. When the sequence of the synthetic gene was examined in conventional manner by means of a DNA sequencing system (370A, ABI), it could be confirmed that ligation was performed in the order as initially designed.

It takes about one day for synthesis of an oligonucleotide of about 100 bases length. For preparation of the same

synthetic gene as in Reference Example 6 according to the prior art method, in addition to the three oligonucleotides of 100 bases length synthesized in Example 6, oligonucleotides of 92, 84 bases length must be synthesized. For the purpose, at least 2 superfluous days are required for synthesis. Besides, purification are also required.

According to the method of the Reference Example, in the case of preparing a synthetic gene of the same length as in the prior art method, it could be prepared with about half time and about half of the reagents.

Reference Example 7

In addition to the three oligonucleotides used in Reference Example 6, further the three oligonucleotides shown below were synthesized, and by use of 6 oligonucleotides as the total, the same experiment as in Example 6 was conducted.

4) 5' TGACGGTTCATCGGTTCTAAATTC

CGTCACGAGCGTACCATACTAATTGG

ATCTACTGGGCGCGGTACGCTGACTG

GCTGTTCACCAACGCCGCTGTTGTT 3'

5) 5' AACCGTCATCGCGTTGCGCGTTGA

CGCGGATCAGGGAACGGTTAGACCTC

GCGTTGCTCGTTCAACGCCGATCAGGG

AACGATCTACTGCGCGGTACGC 3'

6) 5' TGAGAATTTCGCGATCTTCGGCGAA

GCCGAAGCGCCGGAGCTGAGCGTACC

G 3'

In the first synthetic oligonucleotide described in Reference Example 6 and the sixth synthetic oligonucleotide, the restriction enzyme EcoRI site is incorporated. Also, 8 base pairs from the 3'-end or the 5'-end (underlined portions) are complementary to each other.

A part of the oligonucleotide synthesized was examined for its purity by 10 % polyacrylamide gel electrophoresis containing 7 M urea to be judged as having a purity of about 10 %, and therefore a band with desired length was cut out from the gel, and the desired oligonucleotides were extracted with 1 mM EDTA, recovered by ethanol precipitation and then used in the following reactions.

Each oligonucleotide in an amount of 0.1 µg was placed in an Eppendorf tube, 10 µl of 10 x kinase buffer (0.5M Tris-HCl pH 8.0 ; 0.1M MgCl₂ ; 0.1M β-mercaptoethanol), 10 µl of 1 mM ATP and 1 µl of T4 polynucleotide kinase (10 units) (TOYOBO) were added, and the mixture was heated at 37 °C for one hour.

Next, 5 µl of 10 x annealing solution (100 mM Tris-HCl pH 8.0 ; 60 mM MgCl₂ ; 60 mM β-mercaptoethanol ; 500 mM NaCl) was added, and the mixture was made up to 50 µl with distilled water.

These oligonucleotides were heated in a beaker containing 200 ml of hot water of 65 °C for 10 minutes, and then left to cool slowly to room temperature. By this reaction, the six oligonucleotides were annealed as shown in Fig. 3.

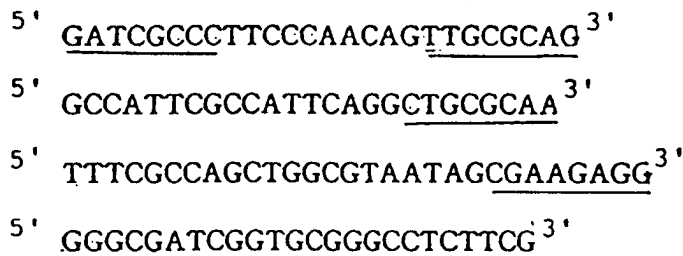
Next, into 50 µl of the annealed oligonucleotide solution were added 5 µl of 10 x annealing solution, each 2 µl of 1 mM dATP, dCTP, dGTP and TTP, and further distilled water to make the total liquid amount to 100 µl, and then 12 units of Klenow fragment of DNA polymerase I were added, followed by the reaction at 37 °C for one hour to perform the extension reaction.

Further, phenol extraction were performed from the above reaction mixture, the DNA was recovered by ethanol

precipitation and its length was examined by 10 % polyacrylamide gel electrophoresis. As the result, a band was detected at the position of about 500 base pairs. Next, digestion was effected in conventional manner with the restriction enzyme EcoRI, and the synthetic gene was ligated to the EcoRI site of the M13 phage. *E. coli* JM109 was transformed with the ligated product to obtain plaques bearing the plasmid containing the synthetic gene. When the sequence of the synthetic gene was examined in conventional manner by means of a DNA sequencing system (370A, available from ABI), it could be confirmed that ligation was performed in the order as initially designed.

Reference Example 8

Four kinds of oligonucleotides as shown below corresponding to a part of the base sequence of the plasmid pUC19 were synthesized by means of a DNA synthesizer (Model 381A, Applied Biosystems).

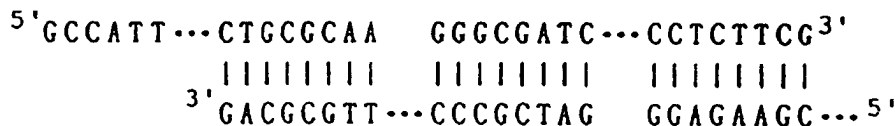


Eight bases from the 3'-end or the 5'-end are complementary to each other.

A part of the oligonucleotide synthesized was examined for its purity by 20 % polyacrylamide gel (containing 7 M urea) electrophoresis. As the result, it was found to have a purity of about 95 %, and therefore used without further purification in the following reactions.

Each oligonucleotide in an amount of 2 µg (about 130 pmole) was placed in an Eppendorf tube, 10 µl of 10 x kinase buffer (0.5M Tris-HCl pH 8.0 ; 0.1M MgCl₂ ; 0.1M β-mercaptoethanol), 10 µl of 1 mM ATP and 1 µl of T4 polynucleotide kinase (10 units) (TOYOBO) were added, and the mixture was heated at 37 °C for one hour.

Next, 5 µl of 10 x annealing solution (100 mM Tris-HCl pH 8.0 ; 60 mM MgCl₂ ; 60 mM β-mercaptoethanol ; 500 mM NaCl) was added, and the mixture was made up to 50 µl with distilled water. This was heated in a beaker containing hot water of 65 °C for 10 minutes, and then left to cool slowly to room temperature (time required is about one hour). By this reaction, the four oligonucleotides form a partial double strand as shown below.



Into 50 µl of this solution were added each 2 µl of 1 mM dATP, dGTP and dCTP, 5 µl of 0.4 mM biotinylated UTP (BRL), 5 µl of 10 x annealing solution, 32 µl of distilled water, and after thorough mixing 16 units of Klenow fragment of DNA polymerase I were added, followed by the reaction at 37 °C for one hour to effect the extension reaction.

Then, for removing the unreacted biotinylated UTP, the reaction mixture was purified by a gel filtration column (Bio-gelP2; Bio Rad, 0.5 x 5 cm). The desired labelled nucleotide was recovered in the fractions which had passed through the column substantially as such.

Each 0.5 ml of the respective fractions was collected, and each 2 µl was adsorbed on a nitrocellulose filter to perform color formation reaction of biotin according to the protocol of BRL. As the result, the second fraction formed the color strongly, whereby it was confirmed that the desired oligonucleotide was labelled with biotin.

Next, comparison was made with the prior art examples (the substitution synthetic method, the nick translation method, and the primer extension method).

A probe with 80 base pairs similar to the above DNA was prepared according to the substitution synthetic method. That is, two oligonucleotides of 80 pairs length were prepared, and after purification by gel electrophoresis (yield of about 20 %) formed into a double strand. The phosphoric acid at its 5'-end was removed, and in place thereof biotinylated dUTP was introduced with a polynucleokinase derived from T4 phage, and the labelled product obtained was dissociated into single strands to provide a probe A. Biotinylated dUTP was incorporated into the double-stranded oligonucleotide of 80 base pairs as mentioned above by nick translation to provide a probe B.

Also, by synthesizing one of the oligonucleotides of 80 bases length as mentioned above, and a primer of 8 bases length complementary thereto at the 3'-end, and after annealing them, the extension reaction with Klenow fragment

was carried out in the same manner as in Reference Example 2 to prepare a probe C (the primer extension method).

Each of the probes A, B, C was purified by gel filtration column in the same manner as mentioned above. In probe A, color formation was observed in the second fraction, but its extent was considerably pale as compared with the present invention. In probe B, substantially no color formation occurred, exhibiting that the reaction had not proceeded well. In probe C, in addition to the second fraction, color formation also occurred in the third and fourth fractions, exhibiting that considerable amounts of by-products with shorter lengths were also formed.

From these facts, it has been shown that the nick translation method and the primer extension method are unsuitable for preparation of a probe of 80 base pairs.

Next, after denaturation of the plasmid DNA pUC19 and pBR322 with an alkali, DNA's in amounts of the group I No. 1 to 10, the group II No. 1 to 10 as shown in Fig. 4 were spotted on a nitrocellulose filter.

Two sheets of such filter were prepared, and after baking to 80 °C for 2 hours, hybridization reactions were carried out by use of two kinds of probes (the substitution synthetic method which is the prior art and that used for the present invention in this Example 8).

When color formation reaction was carried out in conventional manner, in the probe according the substitution synthetic method, color formation occurred under 5 ng at least in the group I, while no color formation observed in the Control of the group II. In the probe prepared according to the method also used for present invention, color formation occurred under 0.05 ng at least, but no color formation in the Control of the group II.

As the result of this experiment, it could be confirmed that not only the probe preparation method is easier according to the method used for the present invention than according to the prior art methods, but also the detection sensitivity is elevated by 100-fold.

Reference Example 9

Four oligonucleotides each being a part of the DNA sequence of bacteriorhodopsin which is a protein existing in H. Halobium were synthesized by means of a DNA synthesizer.

5' GGGGTATGGCCTCACAATGGTACC.3'

5' TACCCCAGCAGCATCGAGAGGTAC.3'

5' TTCTGCTCCCCACCGAACGGTACC.3'

5' GCAGAACCCCATCTACTGGGCGCG.3'

Eight bases from the 3'-end are complementary to each other.

For a part of these oligonucleotides synthesized, 15 % polyacrylamide gel (containing 7 M urea) electrophoresis was conducted for examination of its purity. As the result, the purity was judged to be about 90 % or more, and therefore each oligonucleotide was used in the following reactions without further purification.

In an Eppendorf tube, 45 ng of each oligonucleotide was placed, and 10 µl of 10 x kinase buffer (0.5 M Tris - HCl pH 8.0; 0.1 M MgCl₂; 0.1M β-mercaptoethanol), 10 µl of 1 mM ATP and 1 µl (10 units) of T4 polynucleotide kinase (TOYOBO) and the mixture was heated at 37 °C for one hour.

Next, 2 µl of 10 x annealing solution (see Example 8) was added and the mixture was made up to 20 µl with distilled water.

The mixture was heated in a beaker containing 200 ml of hot water of 65 °C for 10 minutes, then cooled slowly to room temperature. According to this reaction, the four oligonucleotides were annealed.

Next, into 20 µl of the annealed oligonucleotide solution were added 2 µl of 10 x annealing solution, each 100 µCi of ³²P-dATP, ³²P-dCTP, ³²P-dGTP and ³²P-TTP, and further after making up the total liquid amount to 40 µl with addition of distilled water, one unit of Klenow fragment of DNA polymerase I was added, followed by heating at 37 °C for 30 minutes. For removing the unreacted ³²P-TTP from the reaction product, 20 µl of a 98 % formamide-0.05 % xylene cyanol-Bromophenol Blue solution was added into the reaction solution, heated at 90 °C for 2 minutes to denature, followed by 10 % polyacrylamide (containing 7 M urea) gel electrophoresis. After electrophoresis at 1000 V for 3 hours, the gel was removed from the glass plate, and left to stand for about 30 seconds with a X-ray film placed thereon. When this film was developed, and thus a band corresponding to 78 mer was obtained. It was confirmed that the synthetic oligonucleotide of 24 mer became completely the 78 mer according to the extension reaction without observation of other products with shorter lengths. Also, it was confirmed that the extension reaction product was alternately labelled..

The gel corresponding to this position was cut out, and the desired oligonucleotides were extracted overnight at

37 °C with 1 mM EDTA solution, and its intensity was measured by a liquid scintillation counter to find that the probe prepared had a very strong intensity of 10^{10} cpm or higher.

The hybridization reaction by use of this probe gave good results in all of colony, Southern and spot hybridizations.

In the same manner as in Reference Example 8, three kinds of probes of 78 mer were prepared according to the prior art methods. Then, when the unreacted radioactive substance was removed by ethanol precipitation, and the intensity of radiation of the precipitate product was measured by a scintillation counter, an intensity of 10^5 cpm was exhibited in the probe A prepared according to the substitution synthetic method, an intensity of 10^8 cpm was exhibited in the probe B prepared according to the primer extension method and an intensity of 10^3 cpm was exhibited in the probe C prepared according to the nick translation method.

When these probes were examined by gel electrophoresis, although bands were observed at the portion of 78 mer in the probe A and the probe C, a number of bands of shorter by-products were observed in the probe B. Only in this case of probe B, the band corresponding to 78 mer was cut out, subjected to extraction and, ethanol precipitation to recover these probes, and the intensity of radiation was measured to be about 10^6 cpm.

By use of these probes, for the PstI digested product of the whole DNA of *H. Halobium*, Southern hybridization was performed. The electrophoresis of the PstI digested product of 0.01, 0.05, 0.1, 0.5, 1 and 2 μ g in each lane was conducted, after Southern blotting was performed. In the probes A and B, a weak band hybridized with a length of about 5 kb was seen in the lanes electrophorized in 2 μ g and 1 μ g. In the probe C, no band was observed. In the method of the present invention, a strong band was also exhibited at around 5 kb even in a lane of 0.01 μ g, thus showing a detection sensitivity of 100-fold or more of the prior art method.

Reference Example 10

The same experiment as in Reference Example 8 was conducted except for changing dATP, dGTP, dCTP and biotinylated UTP in Reference Example 8 respectively to 2 μ l of 1mM ATP, GTP and CTP, and 5 μ l of 0.4 mM biotinylated UTP.

The two oligonucleotide chains thus prepared had become oligonucleotides in which DNA region and RNA region were alternately incorporated in both chains.

Reference Example 11

The same experiment was conducted in the same manner as in Reference Example 8 except for adding further 2 μ l of 1mM TTP upon adding dATP, dGTP, dCTP.

The Reference Example 11 also exhibited good results.

Example 1

(Capturing probe)

Two kinds of oligonucleotides as shown below corresponding a part of the base sequence of the plasmid pUC19 were synthesized by means of a DNA synthesizer (Model 381A, Applied Biosystems).

5' GATCGCCCTTCCCAACAGTTGCGCAG 3'

5' CATTGCCCATTTCAGGCTGCGCAA 3'

Eight bases from the 3'-end are complementary to each other.

For a part of these oligonucleotides synthesized, 20 % polyacrylamide gel (containing 7 M urea) electrophoresis conducted for examination of its purity. As the result, the purity was found to be about 95 % or more, and therefore each oligonucleotide was used in the following reactions without further purification.

Each oligonucleotide in an amount of 2 μ g (about 130 pmole) was placed in an Eppendorf tube, 5 μ l of 10 x annealing solution (100 mM Tris-HCl, pH 8.0 ; 60 mM, $MgCl_2$; 60 mM β -mercaptoethanol 500 mM NaCl) was added, and the mixture was made up to 50 μ l with distilled water. The mixture was heated in a beaker containing hot water of 65 °C for 10 minutes, then cooled slowly to room temperature (time required was about one hour). According to this reaction, the two oligonucleotides form a partial double strand as shown below.

5' GATCGCCCTTCCCAACAGTTGCGCAG 3'

3' AACGCGTCGGACTTACCGCTTAC 5'

Into 50 μ l of this solution were added each 2 μ l of 1 mM dATP, dGTP, and dCTP, 5 μ l of 0.4 mM biotinylated UTP (BRL), 5 μ l of 10 x annealing solution, 32 μ l of distilled water, and after thorough mixing, 16 units of Klenow fragment of DNA polymerase I (TOYOBO) were added, followed by heating at 37 °C for one hour to perform the extension reaction.

Then, for removing the unreacted biotinylated UTP, the reaction mixture was purified by a gel filtration column (Bio-gelp2; Bio Rad, 0.5 x 5 cm). The desired labelled nucleotide was recovered in the fractions which had passed through the column substantially as such.

Each 0.5 ml of the respective fractions was collected, and each 2 μ l was adsorbed on a nitrocellulose filter to perform color formation reaction of biotin according to the protocol of BRL. As the result, the second fraction formed the color strongly, whereby it was confirmed that biotin was introduced into the desired oligonucleotide.

Accordingly, this fraction was made a capturing probe solution.

(Preparation of nucleic acid for performing detection)

On the other hand, as the nucleic acids for performing detection, the three kinds of PBR322, pUC19 and a mixture thereof were prepared.

In the subsequent description, PBR322 is called Sample A, pUC19 Sample B, and the mixture of pBR 322 and pUC19 Sample C for the purpose of convenience.

Samples A, B, C were prepared according to the same procedure respectively, but in the following, description is made only about Sample A as an example. The samples were labelled.

Into a reaction mixture obtained by adding 1 μ g/2 μ l of a sample DNA into 2.0 μ l of 10-fold TA buffer and, 16.0 μ l H₂O, 10 units of HindIII was added, followed by complete digestion at 37° C for 2 hours. To the product was added 2.0 units of T4 DNA polymerase, and the reaction was carried out at 22 °C for 60 minutes. To the reaction mixture were added each 3.0 μ l of 2 mM dATP, dCTP and dGTP, and further 30 μ Ci of p32-TTP was added to carry out the reaction at 37 °C for 40 minutes. The reaction was stopped with 10 μ l of 100 mM EDTA.

Each 45 μ l of the above probe solution was added into each solution of A, B, C, and distilled water was added thereto to make up 150 μ l.

Then, the reaction mixture was incubated at 50 °C for one hour to carry out the hybridization reaction. After the hybridization reaction, 200 μ l of a 25 w/V % of a Streptoavidin-Agarose suspension at 1M NaCl, 20 mM sodium phosphate (pH 7.5) and 1 mM EDTA was added.

In a rotatory mixer, the biotinylated molecules and the Streptoavidin-Agarose were left to stand at 37° C for 15 minutes so that they could be bound to each other. Agarose was collected by centrifugation for a short time, and the supernatant removed by aspiration. Subsequently, the Agarose was washed once in the above buffered 1M NaCl and twice in a solution containing 150 mM NaCl, 15 mM sodium citrate (pH 8) and 0.2 % SDS at 37 °C

The radioactivity of the precipitate after washing was measured by a scintillation counter for several times to give the results that the biological sample containing pUC19 exhibited a value of 10⁶ to 10⁷ cpm, while the value in the sample containing only pBR322 was less than 2-fold of the background.

From the above description, it was confirmed that the capturing probe according to the present invention fully functioned.

Example 2

Four kinds of oligonucleotides as shown below corresponding a part of the base sequence of the plasmid pUC19 were synthesized by means of a DNA synthesizer (Model 381A, Applied Biosystems).

5' GATCGCCCTTCCCAACAGTTGCGCAG 3'
 5' GCCATTCGCCATTCAGGCTGCGCAA 3'
 5' TTTCGCCAGCTGGCGTAATAGCGAAGAGG 3'
 5' GGGCGATCGGTGCGGGCCTCTTCG 3'

In these sequences, 8 bases from the 3'-end or the 5'-end are complementary to each other.

For a part of these oligonucleotides synthesized, 20 % polyacrylamide gel (containing 7 M urea) electrophoresis was conducted for examination of its purity. As the result, the purity was found to be about 95 % or more, and therefore each oligonucleotide was used in the following reactions without further purification.

Each oligonucleotide in an amount of 2 µg (about 130 pmole) was placed in an Eppendorf tube, 10 µl of 10 x kinase buffer (0.5M Tris-HCl, pH 8.0; 0.1M, MgCl₂; 0.1M β-mercaptoethanol), 10 µl of 1 mM ATP and 1 µl (10 units) of T4 polynucleotide kinase (10 units) (TOYOBO) were added, and the mixture was heated at 37 °C for one hour.

Then, 5 µl of 10 x annealing solution (100 mM Tris-HCl pH 8.0; 60 mM MgCl₂; 60 mM β-mercaptoethanol; 500 mM NaCl) was added, and the mixture was made up to 50 µl with distilled water. The mixture was heated in a beaker containing hot water of 65 °C for 10 minutes, then cooled slowly to room temperature (time required was about one hour). According to this reaction, the four oligonucleotides formed a partial double-strand as shown below.

5' GCCATT ... CTGCGCAA GGGCGATC ... CCTCTTCG
 3' GACGCGTT ... CCCGCTAG GGAGAAGC ...

Into 50 µl of this solution were added each 2 µl of 1 mM dATP, dGTP, and dCTP, 5 µl of 0.4 mM biotinylated UTP (BRL), 5 µl of 10 x annealing solution, 32 µl of distilled water, and after thorough mixing, 16 units of Klenow fragment of DNA polymerase (TOYOBO) were added, followed by heating at 37 °C for one hour to perform the extension reaction.

Then, for removing the unreacted biotinylated UTP, the reaction mixture was purified by a gel filtration column (Bio-gelP2; Bio Rad, 0.5 x 5 cm). The desired labelled nucleotide was recovered in the fractions which had passed through the column substantially as such.

Each 0.5 ml of the respective fractions was collected, and each 2 µl was adsorbed on a nitrocellulose filter to perform color formation reaction of biotin according to the protocol of BRL. As the result, the second fraction formed the color strongly, whereby it was confirmed that biotin had been introduced into the desired oligonucleotide.

Accordingly, this fraction was made a capturing probe solution.

On the other hand, as the nucleic acids for performing detection, the three kinds of pBR322, pUC19 and a mixture thereof were prepared.

In the subsequent description, pBR322 is called Sample A, pUC19 Sample B, and the mixture of pBR 322 and pUC19 Sample C for the purpose of convenience.

Samples A, B, C were prepared according to the same procedure respectively, but in the following, description is made only about Sample A as an example. The samples labelled.

Into a reaction mixture obtained by adding 1 µg/2 µl of a sample DNA into 2.0 µl of 10-fold TA buffer and 16.0 µl H₂O, 10 units of HindIII was added, followed by complete decomposition at 37 °C for 2 hours. To the product were added 2.0 units of T4 DNA polymerase, and the reaction was carried out at 22 °C for 60 minutes. To the reaction mixture were added each 3.0 µl of 2 mM dATP, dCTP and dGTP, and further 30 µCi of p32-TTP was added to carry out the reaction at 37 °C for 40 minutes. The reaction was stopped with 10 µl of 100 mM EDTA.

Each 45 µl of the above probe solution was added into each solution of A, B, C, and distilled water was added thereto to make up 150 µl.

Then, the reaction mixture was incubated at 50 °C for one hour to carry out the hybridization reaction. After the hybridization reaction, 200 µl of a 25 w/V % of a Streptoavidin-Agarose suspension at 1M NaCl, 20 mM sodium phosphate (pH 7.5) and 1 mM EDTA was added.

In a rotatory mixer, the biotinylated molecules and the Streptoavidin-Agarose were left to stand at 37 °C for 15 minutes so that they could be bound to each other. Agarose was collected by centrifugation for a short time, and the supernatant removed by aspiration. Subsequently, the Agarose was washed once in the above buffered 1M NaCl and twice in a solution containing 150 mM NaCl, 15 mM sodium citrate (pH 8) and 0.2 % SDS at 37 °C.

The radioactivity of the precipitate after washing was measured by a scintillation counter for several times to give

the results that the biological sample containing pUC19 exhibited a value of 10^6 to 10^7 cpm, while the value in the sample containing only pBR322 was less than 2-fold of the background.

From the above description, it could be confirmed that the capturing probe according to the present invention fully functioned.

Example 3

a. Preparation of synthetic oligonucleotide:

Four kinds of oligonucleotides as shown below containing base sequences corresponding a part of the base sequence of the plasmid pUC19 were synthesized by means of a DNA synthesizer (Model 381A, Applied Biosystems).

5' GATCGCCCTTCCCAACAGTTGCGCAG 3'

5' GCCATTGCGCATTTCAGGCTGCGCAA 3'

5' TTTCGCCAGCTGGCGTAATAGCGAAGAGG 3'

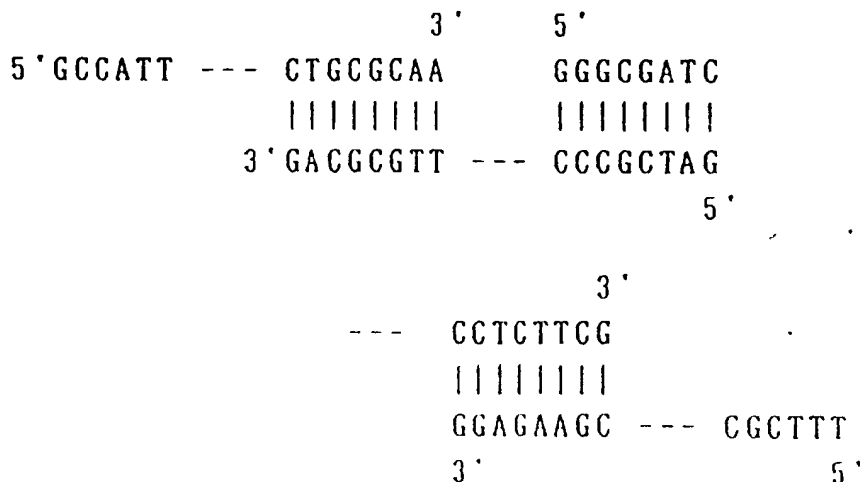
5' GGGCGATCGGTGCGGGCCTCTTCG 3'

Eight bases from the 3'-end or the 5'-end are complementary to each other.

For a part of these oligonucleotides synthesized, 20 % polyacrylamide gel (containing 7 M urea) electrophoresis was conducted for examination of its purity. As the result, the purity was found to be about 95 % or more, and therefore each oligonucleotide was used in the following reactions without further purification.

Each oligonucleotide in an amount of 2 μ g (about 130 pmole) was placed in an Eppendorf tube, 10 μ l of 10 x kinase buffer (0.5M Tris-HCl, pH 8.0; 0.1M, $MgCl_2$; 0.1M β -mercaptoethanol), and 1 μ l (10 units) of T4 polynucleotide kinase (10 units) (TOYOBO) were added, and the mixture was heated at 37 °C for one hour.

Then, 5 μ l of 10 x annealing solution (100 mM Tris-HCl pH 8.0; 60 mM $MgCl_2$; 60 mM β -mercaptoethanol-500 mM NaCl) was added, and the mixture was made up to 50 μ l with distilled water. The mixture was heated in a beaker containing hot water of 65 °C for 10 minutes, then cooled slowly to room temperature (time required was about one hour). According to this reaction, the four oligonucleotides form a partial double strand as shown below.



Into 50 μ l of this solution were added each 2 μ l of 1 mM dATP, dGTP, and dCTP, 5 μ l of 0.4 mM biotinylated UTP (BRL), 5 μ l of 10 x annealing solution, 32 μ l of distilled water, and after thorough mixing, 16 units of Klenow fragment of DNA polymerase I (TOYOBO) were added, followed by heating at 37 °C for one hour to perform the extension reaction. After the reaction, the reaction was stopped by heating at 65 °C for 5 minutes, and then deproteinization was performed by treatment with phenol.

Then, for removing the unreacted biotinylated UTP, the reaction mixture was purified by a gel filtration column (Bio-

gelP2; Bio Rad, 0.5 x 5 cm). The desired capturing modified synthetic nucleotide was recovered in the fractions which had passed through the column substantially as such. Each 0.5 ml of the respective fractions was collected, and each 2 µl was adsorbed on a nitrocellulose filter to perform color formation reaction of biotin according to the protocol of BRL. As the result, the second fraction formed the color strongly, whereby it was confirmed that the desired double-stranded synthetic nucleotide contained biotin.

Next, the solution containing the double-stranded synthetic nucleotide as mentioned above was treated by heating at 95 °C for 5 minutes to be dissociated into single strands, to obtain a synthetic oligonucleotide.

b. Preparation of bound product of synthetic oligonucleotide and insoluble carrier:

As the insoluble carrier, gel particles with particle sizes of about 10 µm were used. The gel particles were obtained by pulverizing Agarose gel, and their surfaces were activated with cyanogen bromide and then bound to avidin. The avidin-bound gel particles were mixed with the solution containing the previous synthetic oligonucleotide for 20 minutes. At this time, the biotin incorporated within the synthetic oligonucleotide binds specifically to the avidin on the surface of gel particle.

c. Preparation of nucleic acid to be detected

As the nucleic acid to be detected, the three kinds of pUC19, pBR322 and a mixture of pUC19 and pBR322 were prepared.

Into a reaction mixture having 2 µl of 10 x TA buffer, 16 µl of ddH₂O added into each sample of the three kinds containing 1 µg of each nucleic acid in 2 µl of distilled water were added 10 units of Hind III, followed by complete digestion at 37 °C for 2 hours. Then, phenol extraction and ethanol precipitation were performed, and the precipitate was dissolved in 10 µl of distilled water.

To the solution were added 6 µl of a mixed reagent solution (0.33M Tris-HCl pH 7.9; 33mM MgCl₂, 3.3 mM dithiothreitol), 1 µl of 5 mM dCTP, 1 µl of 5mM dGTP, 1 µl of 5 mM dATP, 1 µl of 5mM dTTP, 1 µl of [³²P]dGP (10 Ci), 7 µl of distilled water and 2 µl (4 units) of T4-DNA polymerase, and the reaction was carried out at 20 °C for 30 minutes to apply radioactive label. The samples were labelled.

Then, the nucleic acid to be detected of each sample applied with radioactive label was dissociated into single strands by the heat treatment at 95 °C for 5 minutes.

d. Hybridization

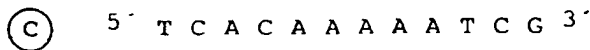
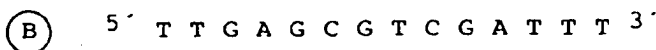
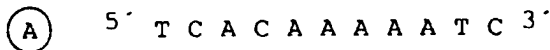
To the bound product of the synthetic oligonucleotide and the insoluble carrier prepared in b. were added the test sample nucleic acid solution dissociated into single strands obtained in c and 5 µl of 10 x annealing solution, and the mixture was made up to 50 µl with distilled water. After the mixture was lightly stirred, it was heated in a beaker containing hot water to 65 °C for 10 minutes, and then cooled slowly for about one hour to room temperature.

After centrifuged briefly, the supernatant was discarded, and after further washing twice with TE solution, the intensity of the precipitate was measured by a scintillation counter for several times. As the result, the sample containing pUC19 exhibited a value of 10⁶ to 10⁷ cpm, and the value in the sample containing only pBR322 was less than 2-fold of the background.

Reference Example 12

a. Preparation of synthetic oligonucleotide

Nine kinds of oligonucleotides as shown below were synthesized by means of a DNA synthesizer (Model 381A, ABI) (designed so that the double-stranded DNA of 20 mer of the final desired product may become the same sequence).



(D) 5' T T G A G C G T C G A T T 3'

5 (E) 5' T T G A G C G T C G A T T T T 3'

(F) 5' T C A C A A A A A T C G A 3'

10 (G) 5' T T G A G C G T C G A T T T T T 3'

15 (H) 5' T C A C A A A A A T C G A C G C T C A A 3'

(I) 5' T T G A G C G T C G A T T T T T G T G A 3'

20 Among them, (A) and (B), (C) and (D), (A) and (E), (D) and (F), (A) and (G), (C) and (E) have sequences at the 3'-ends complementary to each other, (H) and (I) have all the sequences complementary to each other.

For a part of these oligonucleotides synthesized, 20 % polyacrylamide gel (containing 7 M urea) electrophoresis was conducted for examination of its purity. As the result, the purity was found to be about 95 % or more, and therefore each oligonucleotide was used in the following reactions without further purification.

25 Each oligonucleotide in an amount of 2 μ g (about 130 pmole) was placed in an Eppendorf tube, 5 μ l of 10 x annealing solution (100 mM tris-HCl, pH 8.0; 60 mM MgCl₂; 60mM β -mercaptoethanol; 500 mM NaCl), and the mixture was made up to 50 μ l with distilled water. Then, the mixture was heated in a beaker containing hot water of 65 °C for 10 minutes and then cooled slowly to room temperature (time required was about one hour). According to this reaction, the oligonucleotides of (1) (A) and (B), (2) (C) and (D), (3) (A) and (E), (4) (D) and (F), (5) (A) and (G), (6) (C) and (E) form partially double strands with the total hydrogen number of 11 to 16.

30 (1) (A) and (B) total hydrogen bond number at complementary portion: 11

5' T C A C A A A A A T C 3'

35 | | | | |

3' T T T A G C T G C G A G T T 5'

(2) (C) and (D) total hydrogen bond number at complementary portion: 12

40 5' T C A C A A A A A T C G 3'

| | | | |

45 3' T T A G C T G C G A G T T 5'

(3) (A) and (E) total hydrogen bond number at complementary portion: 13

50 5' T C A C A A A A A T C 3'

| | | | |

3' T T T T A G C T G C G A G T T 5'

55 (4) (D) and (F) total hydrogen bond number at complementary portion: 14

5' T C A C A A A A A T C G A 3'

5

3' T T A G C T G C G A G T T 5'

(5) (A) and (G) total hydrogen bond number at complementary portion: 15

10

5' T C A C A A A A A T C 3'

| | | | | | |

15

3' T T T T T A G C T G C G A G T T 5'

(6) (C) and (G) total hydrogen bond number at complementary portion: 16

20

5' T C A C A A A A A T C G 3'

| | | | | | |

3' T T T T A G C T G C G A G T T 5'

25

On the other hand, (7) H and I, which are all complementary to each other, form the double strand as shown below:

(7) (H) and (I)

30

5' T C A C A A A A A T C G A C G C T C A A 3'

| | | | | | | | | | | | | | | | |

3' A G T G T T T T T A G C T G C G A G T T 5'

35

Into 50 μ l of each solution of (1) to (6) were added each 2 μ l of 1 mM dATP, dGTP, TTP, and dCTP, 5 μ l of 10 x annealing solution, 32 μ l of distilled water, and after thorough mixing, 16 units of Klenow fragment of DNA polymerase I (TOYOBO) were added, followed by heating at 37 °C for one hour to carry out the extension reaction.

40 b. Evaluation

For confirming whether the double-stranded DNA's of (1) to (6) were prepared as intended, after phenol extraction and ethanol precipitation were conducted from the reaction mixtures of a., the precipitate was dissolved in 20 μ l of H₂O, and the 5'-end of the double-stranded DNA of (1) to (7) was labelled with P³².

45

Into an Eppendorf tube of 1.5 ml volume were added 20 μ l of the product of the above a., 4 μ l of a mixed reagent (0.5 M Tris-HCl pH 7.6, 0.1 M MgCl₂, 50 mM dithiothreitol, 1 mM spermidine), 12.5 μ l of [γ -³²P] ATP (125 μ Ci, 3,000 Ci/mmol) and 3 μ l of T4-polynucleotide kinase (12 units), and the reaction was carried out at 37 °C for 30 minutes (the total amount of the reaction mixture 39.5 μ l). After the reaction, 200 μ l of 2.5 M ammonium acetate was added to stop the reaction.

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For removing the unreacted ³²P-ATP from the reaction product, 20 μ l of a 98 % formamide - 0.05 % xylene cyanol - Bromophenol Blue solution was added, and the mixture was heated at 90 °C for 2 minutes to effect modification, followed by 10 % acrylamide gel (containing 7 M urea) electrophoresis. After electrophoresis at 1,000 V for 3 hours, the gel was removed from the glass plate and left to stand for about 30 seconds with an X-ray film placed thereon. When this film was developed, (5) and (6) exhibit clear bands at the same position as (7), while (3) and (4) exhibit weak bands with about 50 % intensity of (7) and further with about 10 % intensity for (1) and (2).

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From the above results, it has been found that a double-stranded oligonucleotide which can be satisfactorily used can be formed according to this reference example, when the total hydrogen bond number at the complementary sequence portion is at least 13.

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Reference Example 13

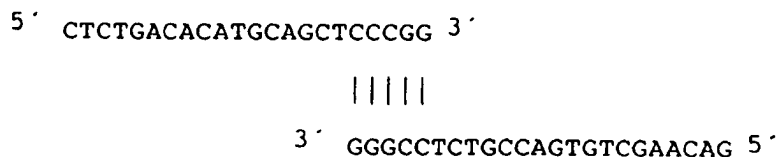
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mixture was made up to 50 μ l with distilled water. Then, the mixture was heated in a beaker containing hot water of

65 °C for 10 minutes and then cooled slowly to room temperature (for about one hour).

According to this reaction, the two oligonucleotides form a partial double strand as shown below:



Into 50 µl of this solution were added each 2 µl of 1 mM dATP, dGTP, TTP, and dCTP, 5 µl of 0.4 mM biotinylated UTP (BRL), 5 µl of 10 x annealing solution, 22 µl of distilled water, and after thorough mixing, 16 units of Klenow fragment of DNA polymerase I (TOYOBO) were added, followed by heating at 37 °C for one hour to carry out the extension reaction.

Then, for removing the unreacted biotinylated UTP, the reaction mixture was purified by a gel filtration column (Bio-gel P2: Bio-Rad, 0.5 x 5 cm). The desired labelled nucleotide was recovered almost in the fractions passed through the column as such.

Each fraction was collected each 0.5 ml, and each 2 µl was adsorbed on a nitrocellulose filter to make color formation reaction according to the protocol of BRL. As the results, the second fraction strongly color formed and then it indicates that these oligonucleotides are labelled with biotin. As shown Table 2, the inventors estimated the extent of color formation in percentage by assuming that strong coloring becomes 2, weak coloring does 1, and no coloring does zero.

From the results as described above, it has been found that an oligonucleotide can be formed provided that the total hydrogen bond number at the complementary sequence portion is a stable sequence having 13 or more.

Further, when the total hydrogen bond number at the complementary sequence portion is 13 to 15, both of those capable of forming oligonucleotides and those of forming no oligonucleotide exist depending on the base sequence of said complementary sequence portion, a number of 16 can be said to be preferable for general purpose.

Table 1

Base number Total hydrogen bond number	4	5	6	7
12	A	-	C	
13		-	D	
14		-	E	H
15		B	F	I
16			G	J

Table 2

Base number Total hydrogen bond number	4	5	6	7
12	20 %	-	30 %	
13		-	60 %	
14		-	70 %	90 %
15		70 %	80 %	100 %
16			100 %	100 %

Reference Example 14

For K to R shown in Table 3, the respective three sets (6 kinds) of the oligonucleotides as shown below were synthesized by a DNA synthesizer (Model 381A, Applied Biosystems). Table 3 shows the combination of the base number and its total hydrogen bond number.

K-1 5' CTCTGACACATGCAGCTCCCCGG 3'
 5' GACAAGCTGTGACCGTCTCCGGG 3'
 K-2 5' CTCTGACACATGCAGCTGCCCCG 3'
 5' GACAAGCTGTGACCGTCTCGGGC 3'
 K-3 5' CTCTGACACATGCAGCTCGCCG 3'
 5' GACAAGCTGTGACCGTCTCGGCG 3'

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L-1 5' AAGGCCAGGAACCGTAAAA 3'
 5' AACGCCAGCAACGCGGCCCTTTTAC 3'
 5 L-2 5' AAGGCCAGGAACCAAGTATT 3'
 5' AACGCCAGCAACGCGGCCAATACT 3'
 10 L-3 5' AAGGCCAGGAACCAAGTAA 3'
 5' AACGCCAGCAACGCGGCCCTTACTT 3'

 M-1 5' TTAAGTTGGGTAAACGCCAGGGTTTT 3'
 15 5' TACAACGTCGTGACTGGGAAAACC 3'
 M-2 5' TTAAGTTGGGTAAACGCCAGATGGTA 3'
 5' TACAACGTCGTGACTGGGTACCAT 3'
 20 M-3 5' TTAAGTTGGGTAAACGCCAGTCAGTT 3'
 5' TACAACGTCGTGACTGGGAACTGA 3'

 N-1 5' GAGAGTGCACCATATGCGGTGTGA 3'
 25 5' CCTTACGCATCTGTGCGGTATTTTACACAC 3'
 N-2 5' GAGAGTGCACCATATGCGTAGCGT 3'
 30 5' CCTTACGCATCTGTGCGGTATTACGCTA 3'
 N-3 5' GAGAGTGCACCATATGCGCTAGAC 3'
 5' CCTTACGCATCTGTGCGGTATTGTCTAG 3'
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 O-1 5' AATTCGAGCTCGGTACCCGGGGAT 3'
 5' CCTGCAGGTCGACTCTAGAGGATCCCC 3'
 O-2 5' AATTCGAGCTCGGTACCCGGGCATC 3'
 40 5' CCTGCAGGTCGACTCTAGAGGGATGCC 3'
 O-3 5' AATTCGAGCTCGGTACCCCGTTGC 3'
 45 5' CCTGCAGGTCGACTCTAGAGGGCAACG 3'

 P-1 5' TGAGTGAGCTAACTCACATTAATT 3'
 50 5' GGGCAGTGAGCGCAACGCAATTAAT 3'
 P-2 5' TGAGTGAGCTAACTCACTATTTTT 3'
 5' GGGCAGTGAGCGCAACGCAAAAATA 3'
 55 P-3 5' TGAGTGAGCTAACTCACTATATAT 3'
 5' GGGCAGTGAGCGCAACGCATATATA 3'

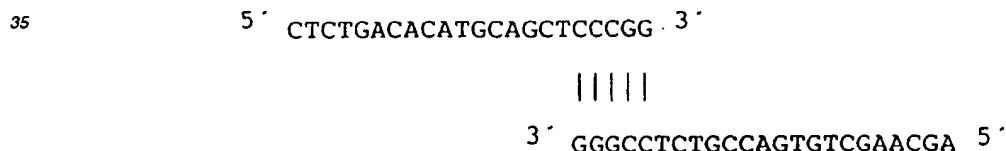


In these sequences, the underlined portions are the 3'-end sequences complementary to each other.

For a part of these oligonucleotides synthesized 20 % polyacrylamide gel (containing 7 M urea) electrophoresis was performed for examination of its purity. As the result, the purity was found to be about 95 % or more, and therefore each oligonucleotide was used in the following reactions without further purification.

Each oligonucleotide in an amount of 2 µg (about 130 pmole) and 5 µl of 10 x annealing solution (100 mM Tris-HCl, pH 8.0; 60 mM MgCl₂; 60 mM β-mercaptoethanol; 500 mM NaCl) were added in an Eppendorf tube, and the mixture was made up to 50 µl with distilled water. Then, the mixture was heated in a beaker containing hot water of 65 °C for 10 minutes and then cooled slowly to room temperature (for about one hour).

According to this reaction, the two oligonucleotides form a partial double strand as shown below:



Into 50 µl of this solution were added each 2 µl of 1 mM dATP, dGTP, TTP, and dCTP, 5 µl of 0.4 mM biotinylated UTP (BRL), 5 µl of 10 x annealing solution, 32 µl of distilled water, and after thorough mixing, 16 units of Klenow fragment of DNA polymerase I (TOYOBO) were added, followed by heating at 37 °C for one hour to carry out the extension reaction.

Then, for removing the unreacted biotinylated UTP, the reaction mixture was purified by a gel filtration column (Bio-gel P2; Bio-Rad, 0.5 x 5 cm). The desired labelled nucleotide was recovered almost in the fractions passed through the column as such.

Each fraction was collected in 0.5 ml, and each 2 µl was adsorbed on a nitrocellulose filter to effect color formation reaction according to the protocol of BRL. The results are shown in Table 4. In Table 4, the oligonucleotides marked "O", the second fraction, formed color strongly, and then these oligonucleotides could be confirmed to be labelled with biotin.

From the above results, it was understood that an oligonucleotide could be formed, provided that the sequence is such as the total hydrogen number of the complementary sequence of 13 or more.

Table 3

<div>Base number</div> <div>Total hydrogen bond number</div>	5	6	7
13	-	L	
14	-	M	P
15	K	N	Q
16		O	R

Table 4

Base number	5	6			7
Total hydrogen bond number		L-1 GTAAAA	X		
		L-2 AGTATT	X		
		L-3 AAGTAA	○		
13	—	M-1 GGTTTT	X	P-1 ATTAATT	○
		M-2 ATGGTA	○	P-2 TATTTT	○
		M-3 TCAGTT	○	P-3 TATATAT	○
14	—	N-1 GTGTGA	X	Q-1 AAAAAATC	○
		N-2 TAGCGT	○	Q-2 TATGTAT	○
		N-3 CTAGAC	○	Q-3 TTAACCT	○
15	—	O-1 GGGGAT	○	R-1 CATAAAG	○
		O-2 GGCATC	○	R-2 AAGATAC	○
		O-3 CGTTGC	○	R-3 TGATCTT	○
16	—				

(The mark "○" indicates those in which color formation was observed, and the mark "X" those in which no color formation was observed)

Next, by use of a plural number of oligonucleotides, the experiments were proceeded based on these results. With reference to the base sequences of the complementary sequence portions marked "O" in Table 4, four kinds of oligonucleotides as shown below were synthesized by a DNA synthesizer (Model 381A, Applied Biosystems).

5' A A G G C C A G G A A C C A A G T A A 3'
 5' C T A G A C A G C A A C G C G G C C T T A C T T 3'
 5' G T C T A G T G G G T A A C G C C A G A T G G T A 3'
 5' T A C A A C G T C G T G A C T G G G T A C C A T 3'

The underlined portions at the 3'-end or the 5'-end are sequences complementary to each other.

The annealing reaction was performed similarly as previously described. According to this reaction, the four oligonucleotides form a partial double strand as shown below.

5' AAGGCC AAGTAA GTCTAG ATGGTA 3'
 ||||| ||||| |||||
 3' TTCATT CAGATC TACCAT CAACAT 5'

Into 50 μ l of this solution were added each 2 μ l of 1 mM dATP, dGTP, TTP, and dCTP, 5 μ l of 0.4 mM biotinylated UTP (BRL), 5 μ l of 10 x annealing solution, 32 μ l of distilled water, and after thorough mixing, 16 units of Klenow fragment of DNA polymerase I (TOYOBO) were added, followed by heating at 37 °C for one hour to carry out the extension reaction.

Then, for removing the unreacted biotinylated UTP, the reaction mixture was purified by a gel filtration column (Bio-gel P2: Bio-Rad, 0.5 x 5 cm). The desired labelled nucleotide was recovered almost in the fractions passed through the column as such.

Each fraction was collected in 0.5 ml, and each 2 μ l was adsorbed on a nitrocellulose filter to make color formation reaction according to the protocol of BRL. As the result, the second fraction formed color strongly, and these oligonucleotides could be confirmed to be labelled with biotin.

Further, when a part of the above color-formed fraction and a part of the color-formed fraction in the sequence B-3 were examined by Agarose electrophoresis, the above oligonucleotide was detected clearly as a single band longer (by about 2-fold) than the oligonucleotide of the sequence B-3, whereby it was confirmed that the extension reaction with the enzyme was completely carried out.

As is apparent from the above Example and Reference Examples according to the present invention, methods with better synthesis yield, smaller byproduct, and hence easier purification are obtained.

Further, a method which can synthesize a gene with a length approximate to 200 base pairs is obtained.

Further, according to the method used for the present invention, since shorter oligonucleotides may be synthesized, and then procedure for purification can be omitted. Also, in the reaction of synthesis by a DNA polymerase, etc. with a single-stranded DNA as the template, it could be confirmed that the reaction could be proceeded in shorter time and more completely than in the prior art method.

In a sense, this method can be also said to be a method for preparing a probe with a length which cannot be prepared by the nick translation method and the primer extension method.

Also, the double-stranded probe oligonucleotide obtained by the present invention has a fixing substance incorporated in both chains, each chain functioning as a probe with highly specific activity, whereby efficiency of hybridization could be enhanced.

Also, according to the present invention, it has become possible to prepare a long and stable capturing probe simply.

Claims

1. A method for separating a target oligonucleotide, comprising:

a) preparing a probe oligonucleotide having a fixing substance by the steps of:

- i) synthesizing at least one pair of oligonucleotides capable of binding with each other through complementary sequences at the 3' - end regions of the oligonucleotides,
- ii) binding the at least one pair of oligonucleotides through the complementary sequences to form partially double-stranded oligonucleotide, and then carrying out an extension reaction in a reaction solution con-

taining a nucleotide to which a fixing substance is bound to polymerise nucleotides onto the partially double-stranded oligonucleotide and to incorporate the fixing substance into the extended part of the double-stranded oligonucleotide obtained by the extension reaction,

iii) denaturing the double-stranded oligonucleotide in which the fixing substance is incorporated to form single-stranded oligonucleotide having the fixing substance ;

b) reacting the single-stranded oligonucleotide having the fixing substance with a target single-stranded oligonucleotide in a mixture of different oligonucleotide to obtain a double-stranded hybrid ;

c) binding the incorporated fixing substance to a carrier which is specifically reactive with the fixing substance ; and

d) separating the hybrid bound to the carrier from the mixture.

2. A method according to Claim 1, wherein the fixing substance is bonded to the carrier before the reaction of the probe oligonucleotide with the target oligonucleotide.

3. A method according to Claim 1, wherein the fixing substance is bonded to the carrier after the reaction of the probe oligonucleotide with the target oligonucleotide.

4. A method for detecting a target oligonucleotide, comprising :

a) preparing a probe oligonucleotide having a fixing substance by the steps of :

i) synthesizing at least one pair of oligonucleotides capable of binding with each other through complementary sequences at the 3'-end regions of the oligonucleotides,

ii) binding the at least one pair of oligonucleotides through the complementary sequences to form partially double-stranded oligonucleotide, and then carrying out an extension reaction in a reaction solution containing a nucleotide to which a fixing substance is bound to polymerise nucleotides onto the partially double-stranded oligonucleotide and to incorporate the fixing substance into the extended part of the double-stranded oligonucleotide obtained by the extension reaction,

iii) denaturing the double-stranded oligonucleotide in which the fixing substance is incorporated to form single-stranded oligonucleotide having the fixing substance ;

b) reacting the single-stranded oligonucleotide having the fixing substance with a target single-stranded oligonucleotide in which a labelling substance is contained to obtain a double-stranded hybrid containing the labelling substance ;

c) binding the incorporated fixing substance to a carrier which is specifically reactive with the fixing substance ;

d) separating the double-stranded hybrid bound to the carrier ; and

e) detecting the labelling substance in the separated double-stranded hybrid.

5. A method according to Claim 4, wherein the fixing substance is bonded to the carrier before the reaction of the probe oligonucleotide with the target oligonucleotide.

6. A method according to Claim 4, wherein the fixing substance is bonded to the carrier after the reaction of the probe oligonucleotide with the target oligonucleotide.

7. A method for separating a target oligonucleotide, comprising :

a) preparing a probe oligonucleotide having a fixing substance by the steps of :

i) synthesizing first and second oligonucleotides capable of binding with each other through complementary sequences at the 3'-end regions of the oligonucleotides, and synthesising third oligonucleotide capable of binding with either of said first or second oligonucleotide through complementary sequences at the 5' - end region of the oligonucleotides,

- ii) binding the first, second and third oligonucleotides through the complementary sequences at the 3'-end and 5'-end regions of the oligonucleotides to form partially double-stranded oligonucleotide, and then carrying out an extension reaction in a reaction solution containing a nucleotide to which a fixing substance is bound to polymerise nucleotides onto the partially double-stranded oligonucleotide and to incorporate the fixing substance into the extended part of the double-stranded oligonucleotide obtained by the extension reaction,
 - iii) denaturing the double-stranded oligonucleotide in which the fixing substance is incorporated to form single-stranded oligonucleotide having the fixing substance ;
 - b) reacting the single-stranded oligonucleotide having the fixing substance with a target single-stranded oligonucleotide in a mixture of different oligonucleotides to obtain a double-stranded hybrid;
 - c) binding the incorporated fixing substance to a carrier which is specifically reactive with the fixing substance ; and
 - d) separating the double-stranded hybrid bound to the carrier from the mixture.
8. A method according to Claim 7, wherein the fixing substance is bonded to the carrier before the reaction of the probe oligonucleotide with the target oligonucleotide.
9. A method according to Claim 7, wherein the fixing substance is bonded to the carrier after the reaction of the probe oligonucleotide with the target oligonucleotide.
10. A method for detecting a target oligonucleotide, comprising :
 - a) preparing a probe oligonucleotide having a fixing substance by the steps of :
 - i) synthesising first and second oligonucleotides capable of binding with each other through complementary sequences at the 3'-end regions of the oligonucleotides, and synthesising third oligonucleotide capable of binding with either of said first or second oligonucleotide through complementary sequences at the 5'-end region of the oligonucleotides,
 - ii) binding the first, second and third oligonucleotides through the complementary sequences at the 3'-end and 5'-end regions of the oligonucleotides to form partially double-stranded oligonucleotide, and then carrying out an extension reaction in a reaction solution containing a nucleotide to which a fixing substance is bound to polymerise nucleotides onto the partially double-stranded oligonucleotide and to incorporate the fixing substance into the extended part of the double-stranded oligonucleotide obtained by the extension reaction,
 - iii) denaturing the double-stranded oligonucleotide in which the fixing substance is incorporated to form single-stranded oligonucleotide having the fixing substance ;
 - b) reacting the single-stranded oligonucleotide having the fixing substance with a target single-stranded oligonucleotide to which labelling substance is incorporated in a mixture of different oligonucleotide to obtain a double-stranded hybrid containing the labelling substance ;
 - c) binding the incorporated fixing substance to a carrier which is specifically reactive with the fixing substance ;
 - d) separating the double-stranded hybrid bound to the carrier from the mixture; and
 - e) detecting the labelling substance in the separated double-stranded hybrid.
11. A method according to Claim 10, wherein the fixing substance is bonded to the carrier before the reaction of the probe oligonucleotide with the target oligonucleotide.
12. A method according to Claim 10, wherein the fixing substance is bonded to the carrier after the reaction of the probe oligonucleotide with the target oligonucleotide.

Patentansprüche

1. Verfahren zur Abtrennung eines Ziel-Oligonukleotids, umfassend:

- 5 a) Das Herstellen einer Oligonukleotid-Sonde mit einer Fixiersubstanz durch die Schritte:
- i) Herstellen mindestens eines Paares von Oligonukleotiden, die in der Lage sind, über komplementäre Sequenzen an den 3'-Endbereichen der Oligonukleotide jeweils aneinander zu binden,
 - 10 ii) Binden des mindestens einen Paares von Oligonukleotiden über die komplementären Sequenzen, um ein teilweise doppelsträngiges Oligonukleotid zu bilden, und dann Ausführen einer Extensionsreaktion in einer Reaktionslösung, die ein Nukleotid, an welches eine Fixiersubstanz gebunden ist, enthält, um Nukleotide auf das teilweise doppelsträngige Oligonukleotid zu polymerisieren und die Fixiersubstanz in den durch die Extensionsreaktion erhaltenen, verlängerten Teil des doppelsträngigen Oligonukleotids einzubauen,
 - 15 iii) Denaturieren des doppelsträngigen Oligonukleotids, in welche die Fixiersubstanz eingebaut ist, um ein einzelsträngiges Oligonukleotid mit der Fixiersubstanz zu bilden;
- b) das Umsetzen des einzelsträngigen Oligonukleotids, welches die Fixiersubstanz besitzt, mit einem einzelsträngigen Ziel-Oligonukleotid in einer Mischung von verschiedenen Oligonukleotiden, um ein doppelsträngiges Hybrid zu erhalten;
- 20 c) das Binden der eingebauten Fixiersubstanz an einen Träger, der mit der Fixiersubstanz spezifisch reaktiv ist; und
- d) das Abtrennen des an den Träger gebundenen Hybrids aus der Mischung.

25 2. Verfahren gemäß Anspruch 1, wobei die Fixiersubstanz vor der Reaktion der Oligonukleotid-Sonde mit dem Ziel-Oligonukleotid an den Träger gebunden wird.

30 3. Verfahren gemäß Anspruch 1, wobei die Fixiersubstanz nach der Reaktion der Oligonukleotid-Sonde mit dem Ziel-Oligonukleotid an den Träger gebunden wird.

4. Verfahren zum Nachweis eines Ziel-Oligonukleotids, umfassend:

- a) Das Herstellen einer Oligonukleotid-Sonde mit einer Fixiersubstanz durch die Schritte:
- 35 i) Herstellen mindestens eines Paares von Oligonukleotiden, die in der Lage sind, über komplementäre Sequenzen an den 3'-Endbereichen der Oligonukleotide jeweils aneinander zu binden,
 - ii) Binden des mindestens einen Paares von Oligonukleotiden über die komplementären Sequenzen, um ein teilweise doppelsträngiges Oligonukleotid zu bilden, und dann Ausführen einer Extensionsreaktion in einer Reaktionslösung, die ein Nukleotid, an welches eine Fixiersubstanz gebunden ist, enthält, um Nukleotide auf das teilweise doppelsträngige Oligonukleotid zu polymerisieren und die Fixiersubstanz in den durch die Extensionsreaktion erhaltenen, verlängerten Teil des doppelsträngigen Oligonukleotids einzubauen,
 - 40 iii) Denaturieren des doppelsträngigen Oligonukleotids, in welche die Fixiersubstanz eingebaut ist, um ein einzelsträngiges Oligonukleotid mit der Fixiersubstanz zu bilden;
- 45 b) das Umsetzen des einzelsträngigen Oligonukleotids, welches die Fixiersubstanz besitzt, mit einem einzelsträngigen Ziel-Oligonukleotid, in welchem eine Markierungssubstanz enthalten ist, um ein doppelsträngiges, die Markierungssubstanz enthaltendes Hybrid zu erhalten;
- c) das Binden der eingebauten Fixiersubstanz an einen Träger, der mit der Fixiersubstanz spezifisch reaktiv ist; und
- 50 d) das Abtrennen des an den Träger gebundenen, doppelsträngigen Hybrids; und
- e) der Nachweis der Markierungssubstanz in dem abgetrennten, doppelsträngigen Hybrid.

55 5. Verfahren gemäß Anspruch 4, wobei die Fixiersubstanz vor der Reaktion der Oligonukleotid-Sonde mit dem Ziel-Oligonukleotid an den Träger gebunden wird.

6. Verfahren gemäß Anspruch 4, wobei die Fixiersubstanz nach der Reaktion der Oligonukleotid-Sonde mit dem Ziel-Oligonukleotid an den Träger gebunden wird.

7. Verfahren zur Abtrennung eines Ziel-Oligonukleotids, umfassend:

a) das Herstellen einer Oligonukleotid-Sonde mit einer Fixiersubstanz durch die Schritte:

- 5 i) Synthetisieren von ersten und zweiten Oligonukleotiden, die in der Lage sind, über komplementäre Sequenzen an den 3'-Endbereichen der Oligonukleotide jeweils aneinander zu binden, und Synthetisieren eines dritten Oligonukleotids, welches in der Lage ist, über komplementäre Sequenzen am 5'-Endbereich der Oligonukleotide entweder an das erste oder das zweite Oligonukleotid zu binden,
- 10 ii) Binden der ersten, zweiten und dritten Oligonukleotide über die komplementären Sequenzen an den 3'-End- und 5'-Endbereichen der Oligonukleotide, um teilweise doppelsträngiges Oligonukleotid zu bilden, und dann Ausführen einer Extensionsreaktion in einer Reaktionslösung, die ein Nukleotid enthält, an das eine Fixiersubstanz gebunden ist, um Nukleotide auf das teilweise doppelsträngige Oligonukleotid zu polymerisieren und die Fixiersubstanz in den durch die Extensionsreaktion erhaltenen, verlängerten Teil des doppelsträngigen Oligonukleotids einzubauen,
- 15 iii) Denaturieren des doppelsträngigen Oligonukleotids, in dem die Fixiersubstanz eingebaut ist, um einzelsträngiges Oligonukleotid zu bilden, welches die Fixiersubstanz besitzt;
- b) das Umsetzen des einzelsträngigen Oligonukleotids, welches die Fixiersubstanz aufweist, mit einem einzelsträngigen Ziel-Oligonukleotid in einer Mischung von verschiedenen Oligonukleotiden, um ein doppelsträngiges Hybrid zu erhalten;
- 20 c) das Binden der eingebauten Fixiersubstanz an einen Träger, welcher mit der Fixiersubstanz spezifisch reaktiv ist; und
- d) das Abtrennen des an Träger gebundenen doppelsträngigen Hybrids aus der Mischung.

25 8. Verfahren gemäß Anspruch 7, wobei die Fixiersubstanz vor der Reaktion der Oligonukleotid-Sonde mit dem Ziel-Oligonukleotid an den Träger gebunden wird.

9. Verfahren gemäß Anspruch 7, wobei die Fixiersubstanz nach der Reaktion der Oligonukleotid-Sonde mit dem Ziel-Oligonukleotid an den Träger gebunden wird.

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10. Verfahren zum Nachweis eines Ziel-Oligonukleotids, umfassend:

a) das Herstellen einer Oligonukleotid-Sonde mit einer Fixiersubstanz durch die Schritte:

- 35 i) Synthetisieren von ersten und zweiten Oligonukleotiden, die in der Lage sind, über komplementäre Sequenzen an den 3'-Endbereichen der Oligonukleotide jeweils aneinander zu binden, und Synthetisieren eines dritten Oligonukleotids, welches in der Lage ist, über komplementäre Sequenzen am 5'-Endbereich der Oligonukleotide entweder an das erste oder das zweite Oligonukleotid zu binden,
- 40 ii) Binden der ersten, zweiten und dritten Oligonukleotide über die komplementären Sequenzen an den 3'-End- und 5'-Endbereichen der Oligonukleotide, um teilweise doppelsträngiges Oligonukleotid zu bilden, und dann Ausführen einer Extensionsreaktion in einer Reaktionslösung, die ein Nukleotid enthält, an das eine Fixiersubstanz gebunden ist, um Nukleotide auf das teilweise doppelsträngige Oligonukleotid zu polymerisieren und die Fixiersubstanz in den durch die Extensionsreaktion erhaltenen, verlängerten Teil des doppelsträngigen Oligonukleotids einzubauen,
- 45 iii) Denaturieren des doppelsträngigen Oligonukleotids, in dem die Fixiersubstanz eingebaut ist, um einzelsträngiges Oligonukleotid zu bilden, welches die Fixiersubstanz besitzt;
- b) das Umsetzen des einzelsträngigen Oligonukleotids, welches die Fixiersubstanz besitzt, mit einem einzelsträngigen Ziel-Oligonukleotid, in welchem eine Markierungssubstanz enthalten ist, in einer Mischung von verschiedenen Oligonukleotiden, um ein doppelsträngiges, die Markierungssubstanz enthaltendes Hybrid zu erhalten;
- 50 c) das Binden der eingebauten Fixiersubstanz an einen Träger, der mit der Fixiersubstanz spezifisch reaktiv ist; und
- d) das Abtrennen des an den Träger gebundenen, doppelsträngigen Hybrids; und
- 55 e) der Nachweis der Markierungssubstanz in dem abgetrennten, doppelsträngigen Hybrid.

11. Verfahren gemäß Anspruch 10, wobei die Fixiersubstanz vor der Reaktion der Oligonukleotid-Sonde mit dem Ziel-Oligonukleotid an den Träger gebunden wird.

12. Verfahren gemäß Anspruch 10, wobei die Fixiersubstanz nach der Reaktion der Oligonukleotid-Sonde mit dem Ziel Oligonukleotid an den Träger gebunden wird.

5 Revendications

1. Méthode pour séparer un oligonucléotide cible, comprenant :

10 a) la préparation d'un oligonucléotide servant de sonde, comportant une substance de fixation, par les étapes consistant :

- 15 i) à synthétiser au moins une paire d'oligonucléotides pouvant se lier l'un à l'autre par des séquences complémentaires aux régions terminales 3' des oligonucléotides,
 ii) à lier ladite paire au moins d'oligonucléotides par les séquences complémentaires pour former un oligonucléotide partiellement bicaténaire, puis à conduire une réaction d'extension dans une solution réactionnelle contenant un nucléotide auquel une substance de fixation est liée pour polymériser des nucléotides sur l'oligonucléotide partiellement bicaténaire et pour incorporer la substance de fixation dans la partie étendue de l'oligonucléotide bicaténaire obtenu par la réaction d'extension,
 20 iii) à dénaturer l'oligonucléotide bicaténaire auquel la substance de fixation est incorporée pour former un oligonucléotide monocaténaire comprenant la substance de fixation ;

- b) la réaction de l'oligonucléotide monocaténaire comprenant la substance de fixation avec un oligonucléotide monocaténaire cible dans un mélange d'oligonucléotides différents pour obtenir un hybride bicaténaire ;
 25 c) la liaison de la substance de fixation incorporée à un support qui est spécifiquement réactif avec la substance de fixation ; et
 d) la séparation de l'hybride lié au support à partir du mélange.

2. Méthode suivant la revendication 1, dans lequel la substance de fixation est liée au support avant la réaction de l'oligonucléotide servant de sonde avec l'oligonucléotide cible.

3. Méthode suivant la revendication 1, dans lequel la substance de fixation est liée au support après la réaction de l'oligonucléotide servant de sonde avec l'oligonucléotide cible.

4. Méthode pour la détection d'un oligonucléotide cible, comprenant :

35 a) la préparation d'un oligonucléotide servant de sonde et comprenant une substance de fixation par les étapes consistant :

- 40 i) à synthétiser au moins une paire d'oligonucléotides pouvant se lier l'un à l'autre par des séquences complémentaires aux régions terminales 3' des oligonucléotides,
 ii) à lier ladite paire au moins d'oligonucléotides par les séquences complémentaires pour former un oligonucléotide partiellement bicaténaire, puis à conduire une réaction d'extension dans une solution réactionnelle contenant un nucléotide auquel une substance de fixation est liée pour polymériser des nucléotides sur l'oligonucléotide partiellement bicaténaire et pour incorporer la substance de fixation à la partie étendue de l'oligonucléotide bicaténaire obtenue par la réaction d'extension,
 45 iii) à dénaturer l'oligonucléotide bicaténaire auquel la substance de fixation est incorporée pour former un oligonucléotide monocaténaire comprenant la substance de fixation ;

- b) la réaction de l'oligonucléotide monocaténaire comprenant la substance de fixation avec un oligonucléotide monocaténaire cible auquel est incorporée une substance de marquage pour obtenir un hybride bicaténaire contenant la substance de marquage ;
 50 c) la liaison de la substance de fixation incorporée à un support qui est spécifiquement réactif avec la substance de fixation ;
 d) la séparation de l'hybride bicaténaire lié au support ; et
 55 e) la détection de la substance de marquage de l'hybride bicaténaire séparé.

5. Méthode suivant la revendication 4, dans laquelle la substance de fixation est liée au support avant la réaction de l'oligonucléotide servant de sonde avec l'oligonucléotide cible.

6. Méthode suivant la revendication 4, dans laquelle la substance de fixation est liée au support après la réaction de l'oligonucléotide servant de sonde avec l'oligonucléotide cible.

7. Méthode pour la séparation d'un oligonucléotide cible, comprenant :

a) la préparation d'un oligonucléotide servant de sonde et comprenant une substance de fixation par les étapes consistant :

i) à synthétiser des premier et deuxième oligonucléotides pouvant se lier l'un à l'autre par des séquences complémentaires aux régions terminales 3' des oligonucléotides, et à synthétiser un troisième oligonucléotide pouvant se lier à l'un des premier et deuxième oligonucléotides par des séquences complémentaires au niveau de la région terminale 5' des oligonucléotides,

ii) à lier les premier, deuxième et troisième oligonucléotides par les séquences complémentaires aux régions terminales 3' et 5' des oligonucléotides pour former un oligonucléotide partiellement bicaténaire, puis à conduire une réaction d'extension dans une solution réactionnelle contenant un nucléotide auquel une substance de fixation est liée pour polymériser des nucléotides sur l'oligonucléotide partiellement bicaténaire et pour incorporer la substance de fixation à la partie étendue de l'oligonucléotide bicaténaire obtenu par la réaction d'extension,

iii) à dénaturer l'oligonucléotide bicaténaire auquel la substance de fixation est incorporée pour former un oligonucléotide monocaténaire comprenant la substance de fixation ;

b) la réaction de l'oligonucléotide monocaténaire comprenant la substance de fixation avec un oligonucléotide monocaténaire cible dans un mélange d'oligonucléotides différents pour obtenir un hybride bicaténaire ;

c) la liaison de la substance de fixation incorporée à un support qui est spécifiquement réactif avec la substance de fixation ; et

d) la séparation de l'hybride bicaténaire liée au support à partir du mélange.

8. Méthode suivant la revendication 7, dans laquelle la substance de fixation est liée au support avant la réaction de l'oligonucléotide servant de sonde avec l'oligonucléotide cible.

9. Méthode suivant la revendication 7, dans laquelle la substance de fixation est liée au support après la réaction de l'oligonucléotide servant de sonde avec l'oligonucléotide cible.

10. Méthode pour la détection d'un oligonucléotide cible, comprenant :

a) la préparation d'un oligonucléotide servant de sonde comprenant une substance de fixation par les étapes consistant :

i) à synthétiser des premier et deuxième oligonucléotides pouvant se lier l'un à l'autre par des séquences complémentaires aux régions terminales 3' des oligonucléotides, et à synthétiser un troisième oligonucléotide pouvant se lier à l'un des premier et deuxième oligonucléotides par des séquences complémentaires au niveau de la région terminale 5' des oligonucléotides,

ii) à lier les premier, deuxième et troisième oligonucléotides par les séquences complémentaires au niveau des régions terminales 3' et 5' des oligonucléotides pour former un oligonucléotide partiellement bicaténaire, puis à conduire une réaction d'extension dans une solution réactionnelle contenant un nucléotide auquel une substance de fixation est liée pour polymériser des nucléotides sur l'oligonucléotide partiellement bicaténaire et pour incorporer la substance de fixation à la partie étendue de l'oligonucléotide bicaténaire obtenu par la réaction d'extension,

iii) à dénaturer l'oligonucléotide bicaténaire auquel la substance de fixation est incorporée pour former un oligonucléotide monocaténaire comprenant la substance de fixation ;

b) la réaction de l'oligonucléotide monocaténaire comprenant la substance de fixation avec un oligonucléotide monocaténaire cible auquel une substance de marquage est incorporée dans un mélange d'oligonucléotides différents pour obtenir un hybride bicaténaire contenant la substance de marquage ;

c) la liaison de la substance de fixation incorporée à un support qui est spécifiquement réactif avec la substance de fixation ;

d) la séparation de l'hybride bicaténaire lié au support à partir du mélange ; et

e) la détection de la substance de marquage dans l'hybride bicaténaire séparé.

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11. Méthode suivant la revendication 10, dans laquelle la substance de fixation est liée au support avant la réaction de l'oligonucléotide servant de sonde avec l'oligonucléotide cible.

5 12. Méthode suivant la revendication 10, dans laquelle la substance de fixation est liée au support après la réaction de l'oligonucléotide servant de sonde avec l'oligonucléotide cible.

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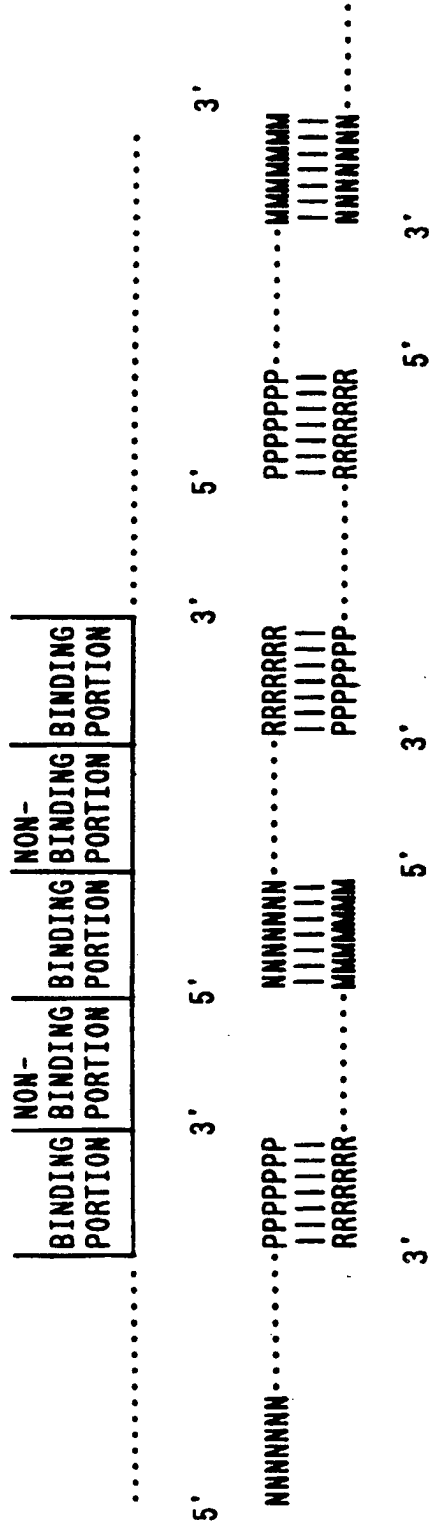
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FIG. 1



M, N, P AND R ARE SELECTED FROM A, T, G AND C, PROVIDED THAT
M AND N, AND P AND R ARE COMPLEMENTARY BASES TO EACH OTHER

FIG. 2



FIG. 3

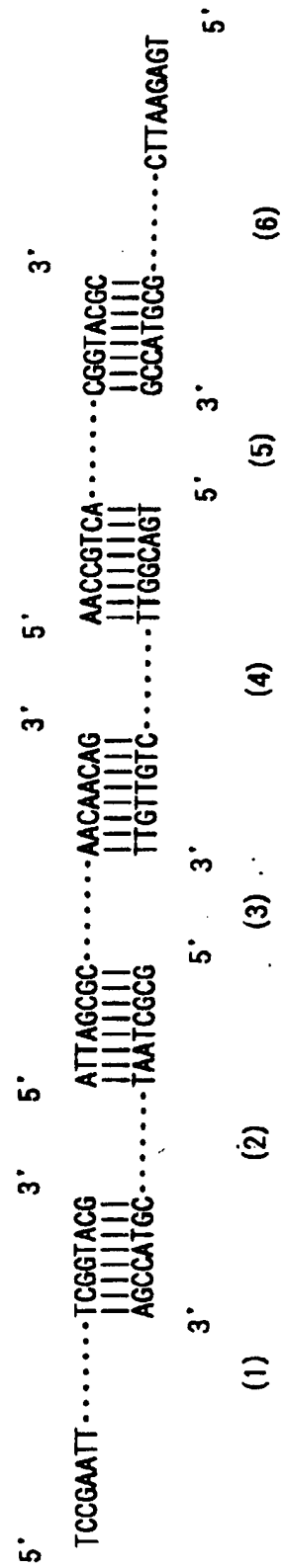
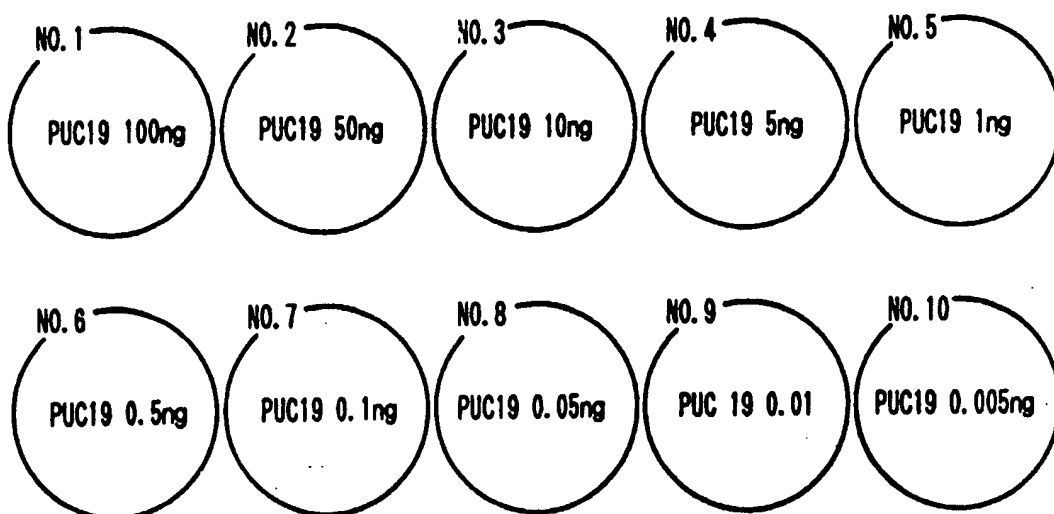


FIG. 4

I GROUP



II GROUP

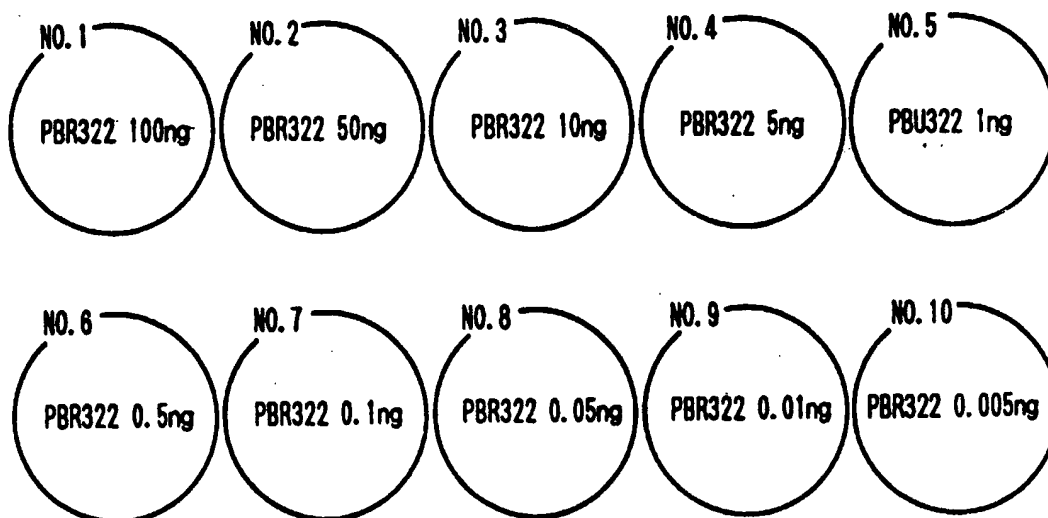


FIG. 5

